Supporting data

Table S1: Settings of critical factors and resulting liposomal characteristics (responses) of the Box–Behnken design.

Run	Critical factors			Responses	
	Bead amount (A) [mg]	Lipid concentration (B) [%]	Homogenization time (C) [min]	Z-Average [nm]	PDI
1	40	20	6	195.2	0.347
2	90	20	3	145.2	0.165
3	40	15	9	142.8	0.227
4	140	15	9	120.5	0.143
5	140	10	6	162.5	0.264
6	90	10	9	144.0	0.218
7	140	20	6	121.1	0.174
8	40	10	6	296.9	0.643
9	40	15	3	249.6	0.530
10	90	15	6	143.7	0.221
11	90	15	6	134.6	0.196
12	90	10	3	216.7	0.468
13	140	15	3	145.2	0.242
14	90	15	6	134.9	0.183
15	90	15	6	140.7	0.225
16	90	15	6	141.3	0.226
17	90	20	9	113.0	0.133

Table S2: Results obtained from ANOVA of the regression model after excluding insignificant terms. The critical factors are coded as A = bead amount, B = lipid concentration, and C = homogenization time. Interaction effects are coded as e.g. AC, quadratic effects as e.g. A^2. F- and p-values reflect the significance of each critical factor on the response.

	Response 1: Z-Average		Response 2: PDI	
Source	F-value	p-value	F-value	p-value
Model	70.04	< 0.0001	36.99	<0.0001
A	149.86	< 0.0001	97.69	<0.0001
B	94.79	< 0.0001	68.73	<0.0001
C	111.29	< 0.0001	53.59	<0.0001
AB	-	-	9.68	0.0144
AC	7.38	0.0217	9.62	0.0146
BC	-	-	10.94	0.0107
A^2	36.84	0.0001	32.81	0.0004
B^2	17.12	0.0020	10.67	0.0114
Lack of Fit	2.47	0.2007	4.73	0.0806

Table S3: Fitting statistics of the Z-Average data (**A**) and PDI (**B**).

A

Std. Dev.	0.0003	**R^2**	0.9768
Mean	0.0066	**Adjusted R^2**	0.9628
C.V. %	4.41	**Predicted R^2**	0.8916
		Adeq Precision	27.8893

B

Std. Dev.	0.0331	**R^2**	0.9737
Mean	0.2708	**Adjusted R^2**	0.9474
C.V. %	12.20	**Predicted R^2**	0.7831
		Adeq Precision	20.1125

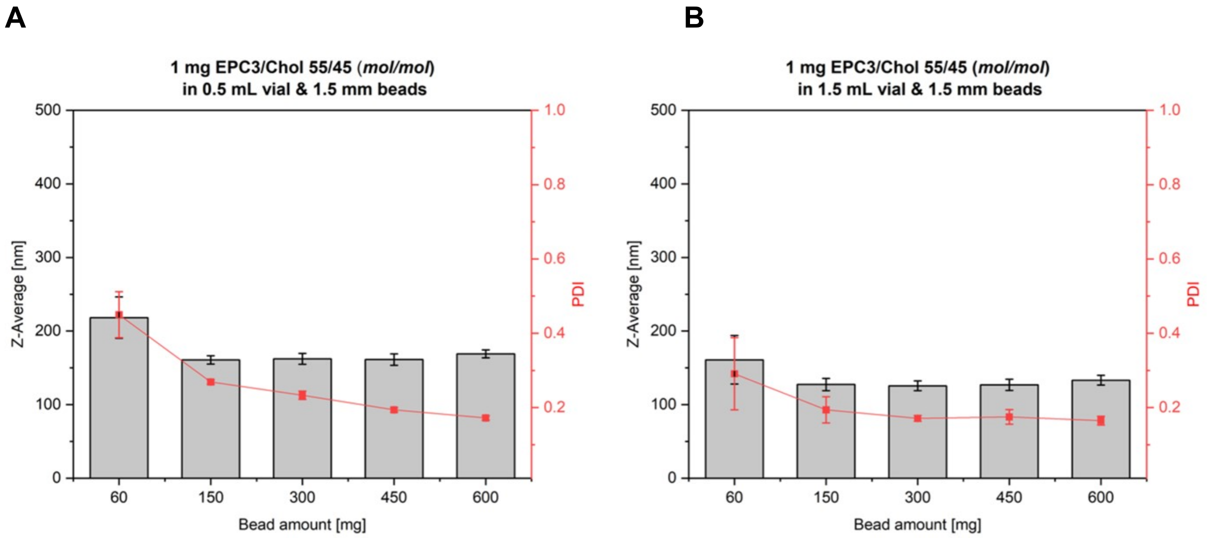


Fig. S1: Particle size (Z-Average) and polydispersity index (PDI) of liposomal dispersion obtained by in-vial homogenization using dual centrifugation (DC) with different vial sizes. A: 0.5 mL vial and B: 1.5 mL vial. Samples were produced from 1 mg EPC3/Chol (55/45) (mol/mol) under the following DC-settings: 2350 rpm, 5 min, 4 ºC followed by redispersion of the vesicular phospholipid gel by DC at 1500 rpm, 2 min, 4 ºC. Bead size (1.5 mm) and lipid concentration (20 %) were kept constant. Different amounts of beads were added in each experiment. Data are presented as mean ± SD (n=3).

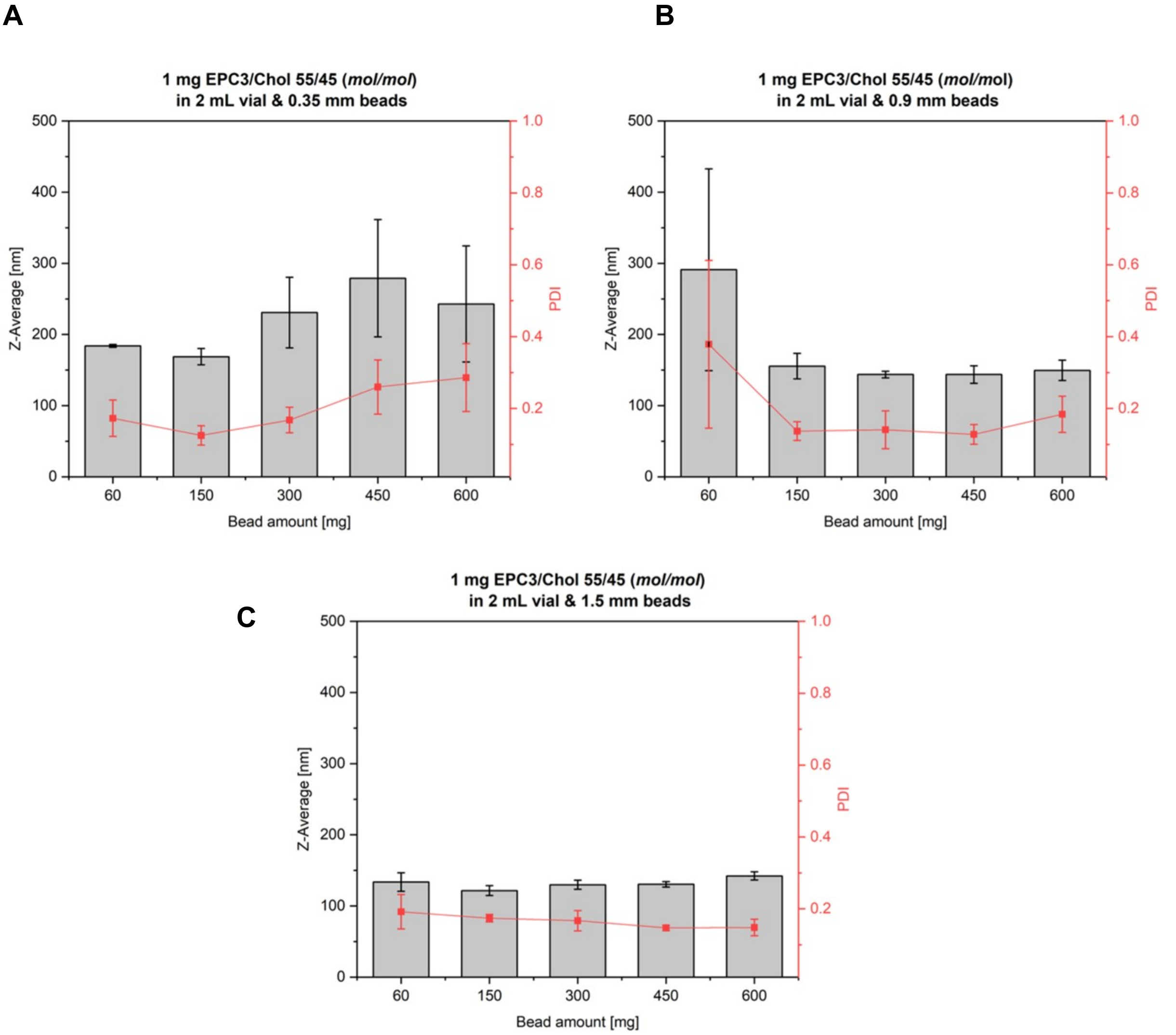


Fig. S2: Particle size (Z-Average) and polydispersity index (PDI) of liposomal dispersions obtained by in-vial homogenization using dual centrifugation (DC) with different bead sizes. A: 0.35 mm beads, B: 0.9 mm beads and C: 1.5 mm beads. Samples were produced from 1 mg EPC3/Chol (55/45) (mol/mol) under the following DC-settings: 2350 rpm, 5 min, 4 ºC followed by redispersion of the vesicular phospholipid gel by DC at 1500 rpm, 2 min, 4 ºC. Vial size (2 mL) and lipid concentration (20 %) were kept constant. Different amounts of beads were added in each experiment. Data are presented as mean ± SD (n=3).

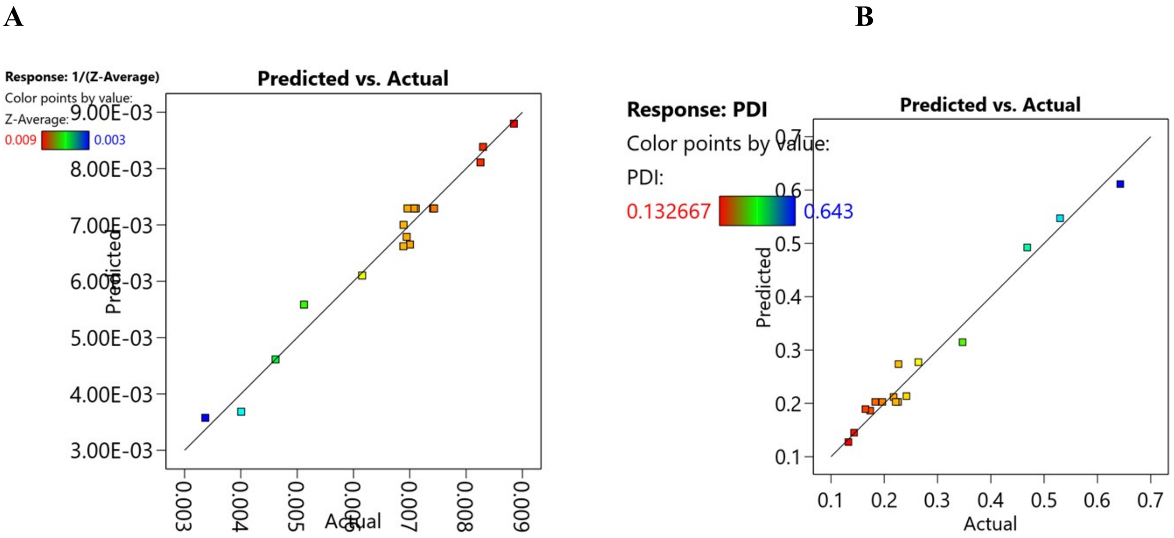


*Fig. S3: Actual versus predicted plots for (**A**) Z-average and (**B**) PDI obtained from the design of experiments (DOE) model.*

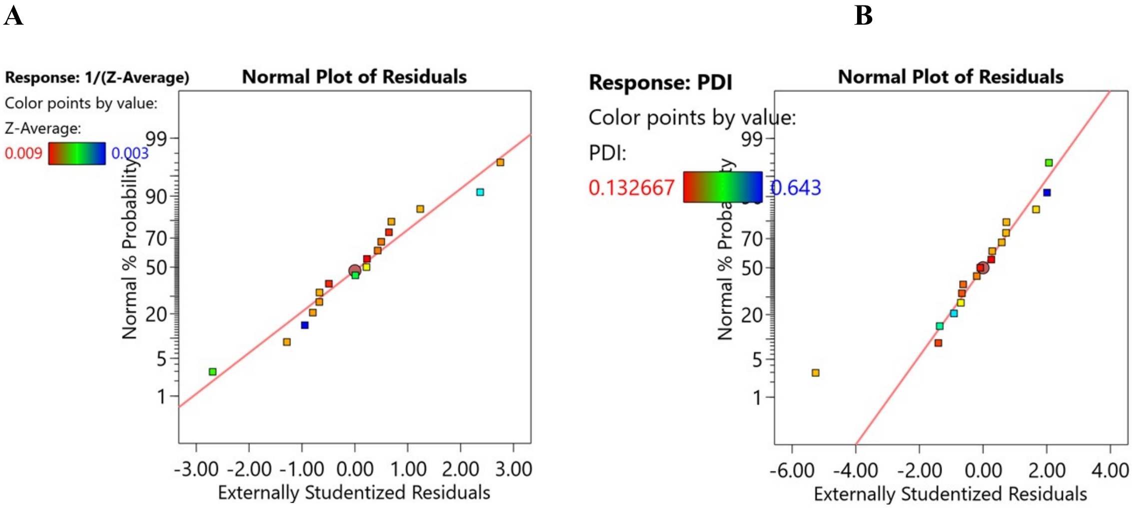


Fig. S4: Normal plots of residuals for (A) Z-average and (B) PDI obtained from the DOE model.

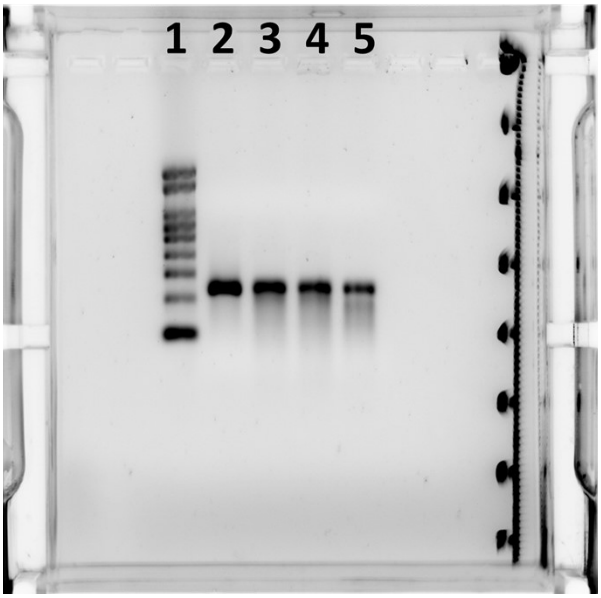


Fig. S5: 1% Agarose gel was performed for integrity studies. The mRNA which undergoes DC in-vial homogenization shows slight degradation as seen in 3, 4, and 5. **1:** RNA ladder; **2:** Positive control (did not undergo proper homogenization); **3:** mRNA loaded LNP - SM-102/DOPE/CHOL/DMG-PEG$_{2000}$ (30/28/40/2); **4:** mRNA loaded LNP - SM-102/DSPC/CHOL/DMG-PEG$_{2000}$ (30/28/40/2); **5:** Blank RNA (underwent same homogenization as the LNPs in absent of lipids).

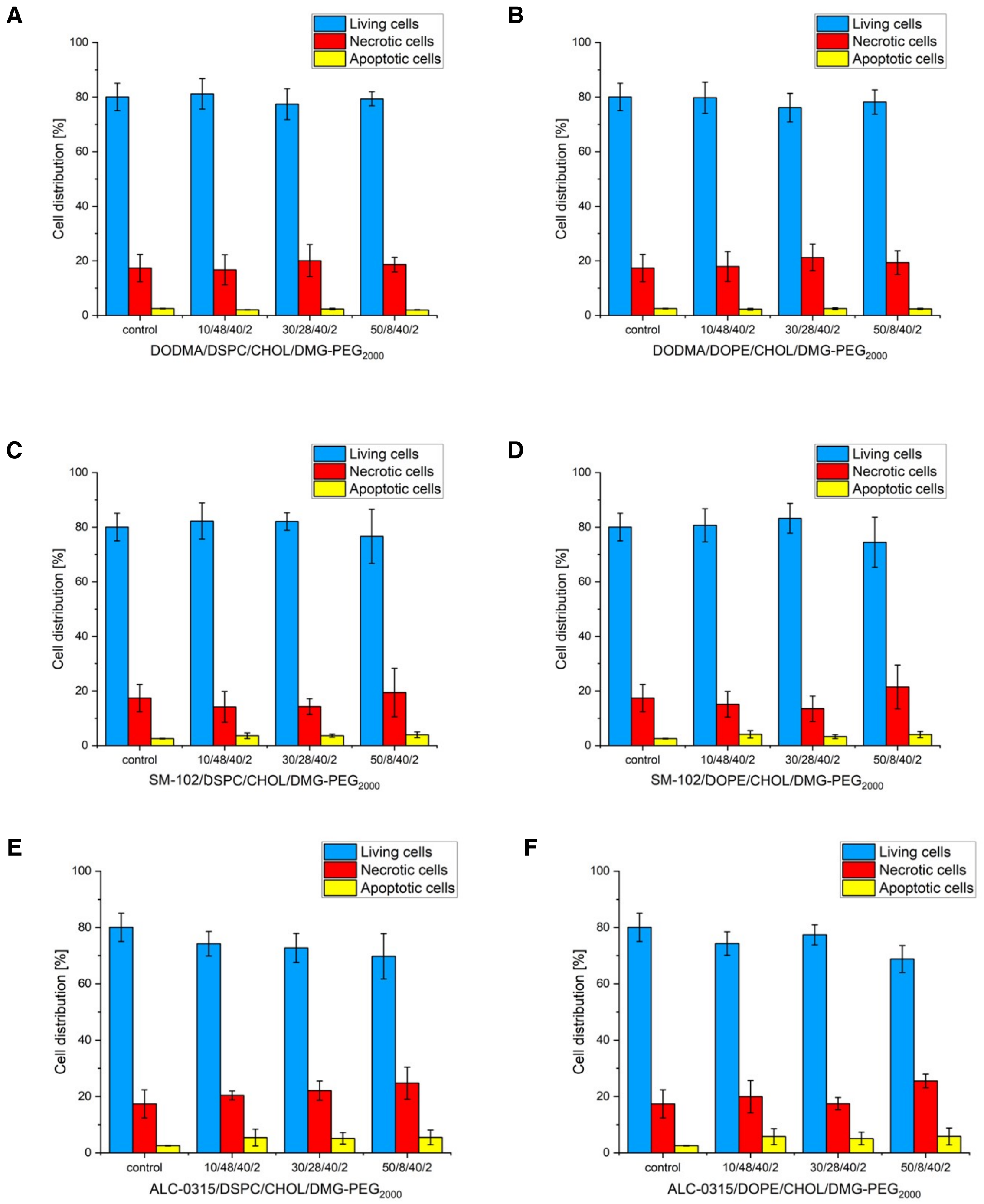


Fig. S6: Cytotoxicity in HEK293A cells evaluated as a percentage of living, necrotic, and apoptotic cells using the CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit. The cells were treated with 75 nM miRNA-100-5p encapsulated in LNPs for 2 h, followed by 72 h of incubation prior to analysis by flow cytometry. For each composition the molar ratio of ionizable lipid was increased while molar ratio of helper phospholipid was decreased. Lipid compositions are expressed as mol%. Data are shown as mean ± SD (n=3).

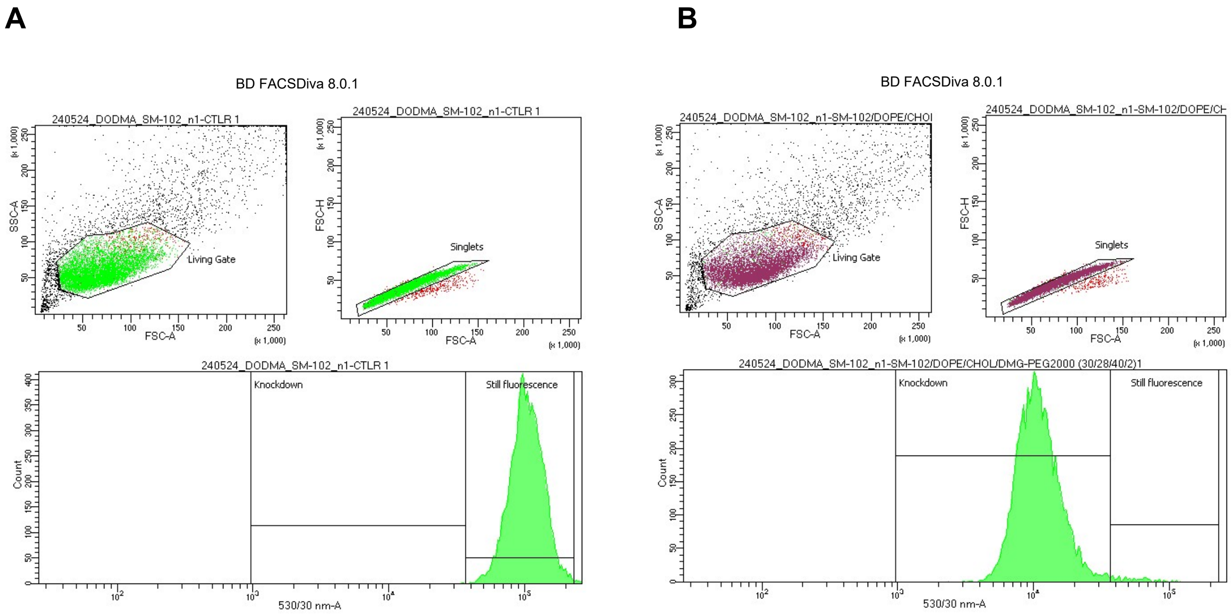


Fig. S7: Gating strategy employed for knockdown measurements via flow cytometry. Knockdown was calculated using the percentage of still ZsGreen positive cells [%]. A = untreated cells (negative control), B = Cells treated with miRNA LNPs comprising SM-102/DOPE/Chol/DMG-PEG$_{2000}$ (30/28/40/2). Lipid composition is displayed as mol%.

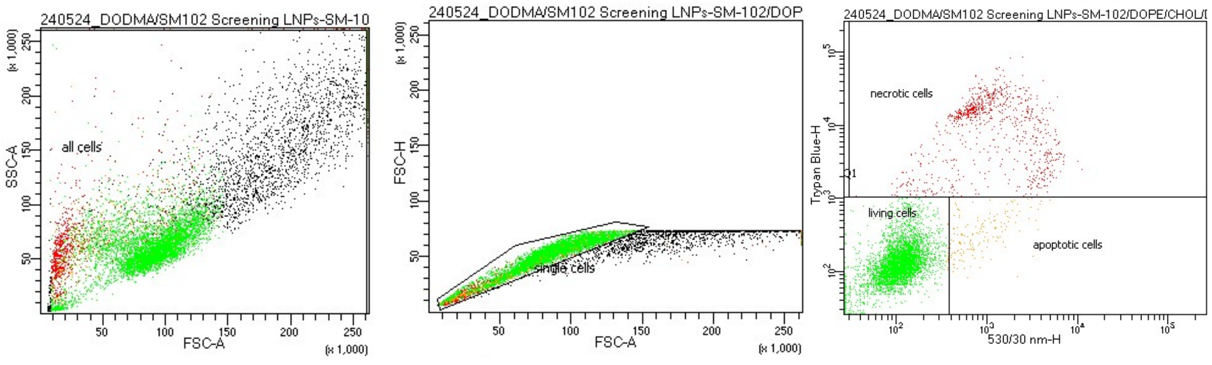


Fig. S8: Gating strategy employed for cytoxicity measurements via flow cytometry. Cytotoxicity was evaluated as a percentage of living, necrotic, and apoptotic cells using the CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit. In this exemplary plot the cells were treated with miRNA LNPs comprising SM-102/DOPE/Chol/DMG-PEG$_{2000}$ (30/28/40/2). Lipid composition is displayed as mol%.

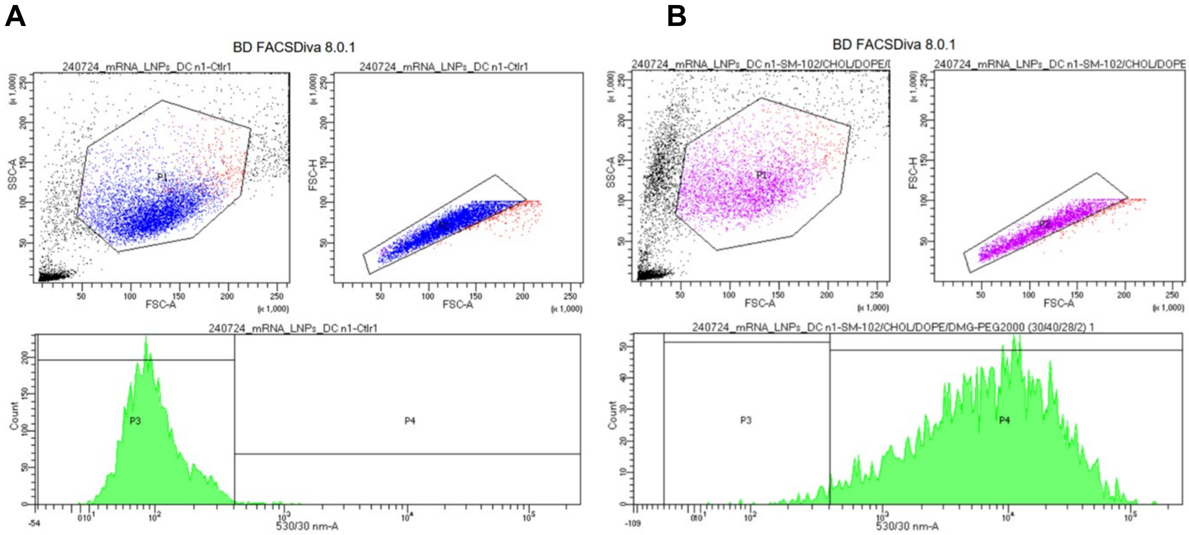
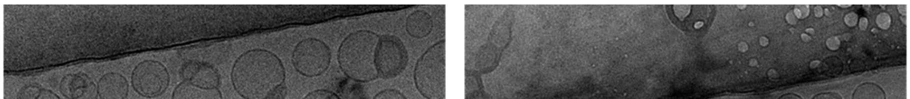


Fig. S9: Gating strategy employed for GFP expression via flow cytometry. GFP expression was calculated using the percentage of GFP positive cells [%] (gate P4). A = untreated cells (negative control), B = Cells treated with mRNA LNPs comprising SM-102/DOPE/Chol/DMG-PEG$_{2000}$ (30/28/40/2). Lipid composition is displayed as mol%.



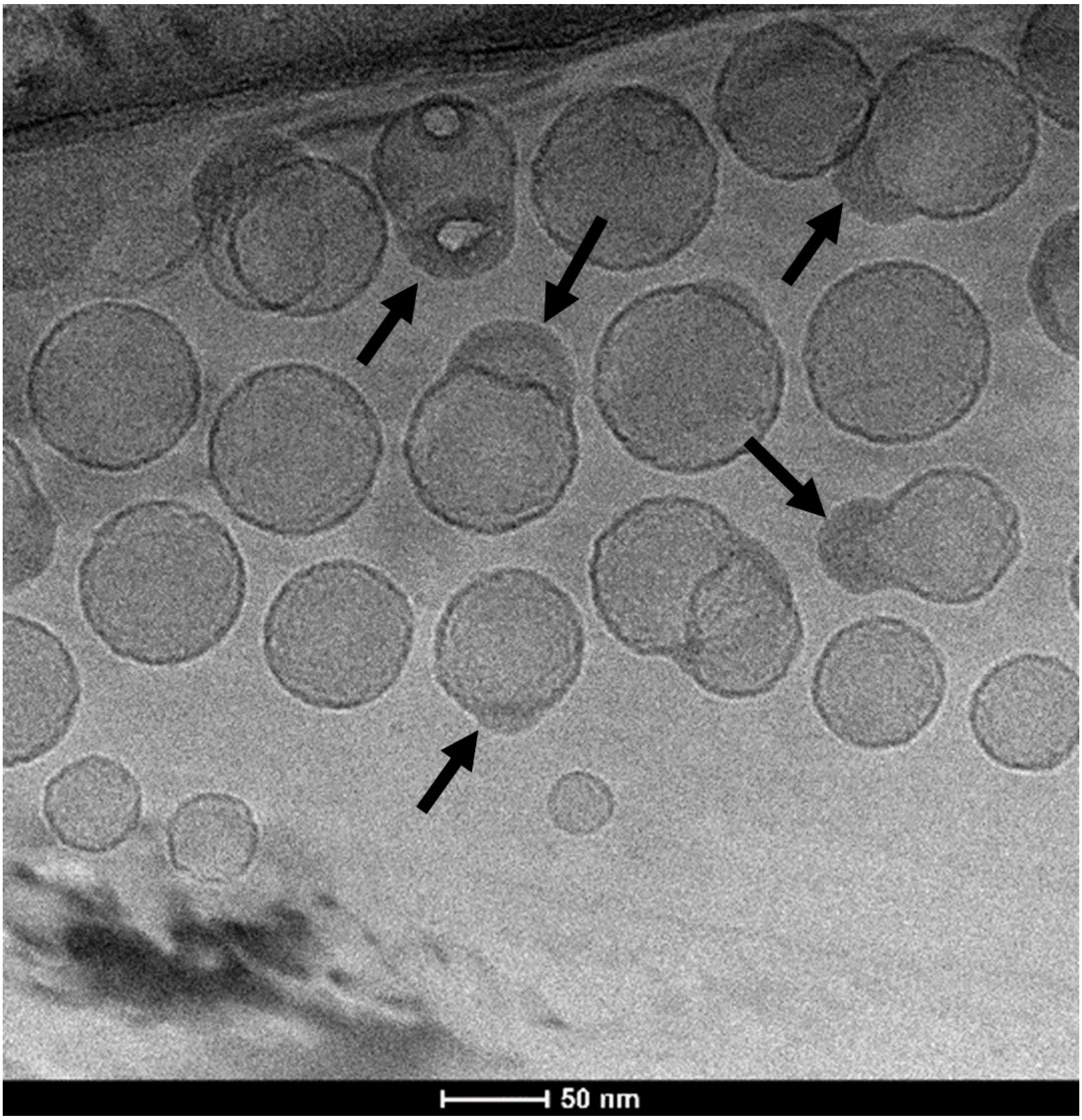


Fig. S10: LNP Morphology investigated by cryo-TEM imaging exhibits non-spherical shapes with characteristic bleb structures. Maximal magnification (DODMA/DSPC/CHOL/DMG-PEG$_{2000}$ 50/8/40/2) reveals a higher density of the blebs suggesting these structures are filled with RNA (indicated by arrows). Sizes of the LNPs are comparable lower as the measured sizes derived from DLS measurements. **A**: DODMA/DSPC/CHOL/DMG-PEG$_{2000}$ (50/8/40/2); **B**: DODMA/DSPC/CHOL/DMG-PEG$_{2000}$ (30/28/40/2); **C**: DODMA/DOPE/CHOL/DMG-PEG$_{2000}$ (50/8/40/2); **D**: DODMA/DOPE/CHOL/DMG-PEG$_{2000}$ (30/28/40/2).