Electronic Supplementary Information (ESI) for

Flash nanoprecipitation assisted self-assembly of ionizable lipid nanoparticles for nucleic acid delivery

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Experimental Section

Materials

Unless specified, all the lipids except cholesterol were purchased from Cayman Chemical (Ann Arbor, MI, USA). Cholesterol was purchased from Alfa Aesar (Haverhill, MA). The Zombie Aqua stain fixable viability kit was obtained from BioLegend (San Diego, California). EZ Cap™ mCherry mRNA and EZ Cap™ Firefly Luciferase mRNA and OVA mRNA were obtained from ApexBio (Houston, Texas). Silencer™ GFP (eGFP) siRNA was obtained from Applied Biosystems (Waltham, MA). SignalSilence® Control siRNA (Cy5® Conjugate) was purchased from Cell Signaling Technology, Inc. (Danvers, MA). NucBlue™ Live ReadyProbes™ Reagent (Hoechst 33342) was obtained from Invitrogen (Waltham, MA). SnakeSkin™ Dialysis Tubing (10 K MWCO) was obtained from Thermo Fisher Scientific Inc (Waltham, MA). mRNA detection kit was obtained from Promega Corporation (Madison, WI). DiD perchlorate was obtained from MedChemExpress (Monmouth Junction, NJ). PE/C5 CD3 antibody, PE/Cy7 CD4 antibody, Pac blue CD45 antibody, Alexa fluor® 488 Ly-6C antibody, and CD16/32 antibodies were obtained from BioLegend (San Diego, California). PerCp-eFluor™ 710 Ly-6G, Super Bright™ 436 CD11c, Super Bright™ 600 F4/80, Super Bright™ 702 CD11b and Super Bright™ CD8a antibodies were obtained from ThermoFisher Scientific (Waltham, MA). Additionally, TNS reagent, 6-p-toludino-2-napthalene-sulfonic acid was obtained from Abcam (Cambridge, United Kingdom). OVA257-264 (SIINFEKL) peptide bound to H-2Kb Antibody was obtained from Invitrogen (Waltham, MA) and OVA peptide (257-264) was obtained from InvivoGen (San Diego, CA).

Animals

All animal use was in accordance with the guidelines and approval from the West Virginia University Institution of Animal Care and Use Committee. Male C57BL/6J mice aged four-six weeks old were well maintained in the animal facility and used for IVIS imaging and *in vivo* uptake studies.

Fabrication of LNPs

LNPs were formed using the flash nanoprecipitation technique using a manual CIJ mixer, as described previously. [1-3] Briefly, based on the molar ratio of all lipids at a 20 mM total lipid calculation, the appropriate amount of lipids (Table S1) were dissolved in 500 µL of an organic solvent such as ethanol or methanol, and nucleic acids were dissolved in 50 mM Sodium citrate buffer (pH 4). The organic and aqueous solvents were impinged against each other to form LNPs in a reservoir containing 2 mL of 50 mM Sodium citrate buffer (pH 4). Organic solvents from the formulations were removed through a 10 KDa dialysis membrane in phosphate-buffered saline (PBS) for 3 h.

Additionally, the conventional ethanol injection method [4, 5] was used to formulate the SM102, ALC0315, and DLin-MC3-DMA LNPs. Briefly, all the lipid components were dissolved in 0.5 mL of absolute ethanol, and mRNA/siRNA was dissolved in 10 mL of sodium citrate buffer (pH 4). A magnetic stirrer (Corning Multiple position 9 x 250 mL) was used to rapidly stir the mRNA sodium citrate solution at 1000 rpm and the ethanolic solution was injected dropwise into the aqueous solution. The formulation was then passed through a 0.22-micron filter and concentrated using Amicon™ Ultra-15 Centrifugal Filter Units from MilliporeSigma, (Burlington, MA).

Determination Of Encapsulation Efficiency

The encapsulation efficiency of RNA was measured using the QuantiFluor® RNA System. Briefly, 1X TE buffered 0.5% Triton X solution was added to the same amount of LNPs encapsulated with RNA at 1:1 dilution. The mixture was then bath sonicated for 90 min at room temperature, followed by probe sonication at 70 amp for 10 seconds on ice. For background subtraction, blank lipid nanoparticles were used for the assay. Initial samples (samples collected just after the LNP formation) and final samples (after dialysis samples) were measured for encapsulation at Ex/Em- 492/540 nm. Relative encapsulation was reported by comparing the concentration of the initial and final samples.

Size and Morphological characterization

The size and surface charge of the LNPs was measured using Dynamic light scattering (DLS) and Electrophoretic light scattering (ELS), respectively on Zetasizer Ultra (Malvern Instruments, UK). All measurements were performed with PBS.

Transmission electron microscopy (TEM) imaging was performed using JEOL 1010 microscope using an AMT XR611S-B CCD camera. LNPs were applied to UV-treated, carbon-coated grids (Ted Pella, 01840-F) and stained immediately with 1% aqueous uranyl acetate before taking the images. Additionally, positive stained TEM images were taken with the JEOL JEM-2100 TEM at 120 KV with the Gatan Camera OneView. Samples were prepared on a copper grid with carbon film and stained using an uranyl acetate solution for two minutes followed by rinsing and dipping in lead citrate solution for four minutes. The dried grid was then utilized for imaging.

TNS Assay

The pKa for LNPs was determined using the 6-p-toludino-2-napthalene-sulfonic acid (TNS, abcam) fluorescent assay. [6, 7] A buffer containing 20 mM sodium monophosphate, 25 mM citrate, 20 mM ammonium acetate, and 150 mM sodium chloride was used to create solutions with a pH ranging from 4.0 to 9.0. Modifications to buffer pH were done by adding sodium hydroxide to the buffer and verifying the pH using the Fisherbrand™ accumet™ AE150 Benchtop pH Meter. For each pH tested, 20 μM total lipid of the LNP along with 6 μM of TNS were added with buffer to create a final volume of 500 μL. Plating was done in triplicate within 96-well black/clear bottom plates (ThermoFisher Scientific, Waltham, MA) where volumes were 150 μL per well. Background wells were also plated in triplicate containing 6 μM TNS within the given pH buffer. Fluorescence was read using SpectraMax iD5 microplate reader with an (Ex/Em - 322/431 nm), respectively. Background values were subtracted from the LNP fluorescent intensities at each pH value. Fluorescent intensities were normalized based on the highest value for the given series. Standard curves were generated using sigmoidal non-linear regressions in Graph-Pad Prism and the pKa for a given LNP was extrapolated at the point where the normalized fluorescent intensity was 0.5.

Cell Culture

RAW 264.7 cells (murine macrophage cell line) were a gift from Hussain Lab, and MDA-MB-231 GFP-expressing cells were obtained from Lockman Lab. DC 2.4 (murine dendritic cells) were obtained from EMD Millipore (Burlington, MA). RAW 264.7 and MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine serum (FBS), penicillin (100 IU/mL) and streptomycin (100 μg/mL) at 37 °C in the presence of air (95%) and CO_2 (5%). In the case of DC 2.4 cells, cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 10% FBS, 1X L-Glutamine, 1X non-essential amino acids, 1X HEPES Buffer Solution and 0.0054X β-Mercaptoethanol at 37 °C in the presence of air (95%) and CO_2 (5%).

***In vitro* Cellular Uptake Studies**

RAW 264.7 cells (2.5 x 10^5 cells/mL) were seeded in 12 well plates and adhered overnight. The adhered cells were incubated with LNPs containing DiD Dye (1 μg/mL) or mRNA (1 μg/mL) for 2, 6, or 24 h. After the incubation period, cells were washed with sterile PBS twice and collected into 5 mL flow cytometer tubes. Apart from single stain controls and no stain control, all the samples were incubated with 50 μL Zombie Aqua (1:500) fixable cell viability dye (Biolegend, San Diego, CA) for 30 min at 4 °C. Cells were washed then with sterile PBS and resuspended in cell staining buffer and analyzed using a BD Fortessa flow cytometer. The cellular uptake was measured as median fluorescent intensity in the APC channel (for DID) and PE-Texas-Red channel (for mCherry mRNA). A similar protocol was followed for the MDA-MB-231 cellular uptake studies with DiD-loaded DLin-MC3-DMA samples.

Confocal Microscopy

RAW 264.7 or DC 2.4 cells (1×10^5 cells/mL, 300 µL) were seeded in each well of an 8-well Chamber slide (iBidi) and kept overnight for adhering. The adhered cells were incubated with LNPs containing DID Dye (5 µg/mL) or mRNA (1 µg/mL) or Cy5 siRNA (10 nM). The LNPs were incubated for 24 h, cells were washed with PBS twice, and NucBlue™ Live ReadyProbes™ Reagent (nuclei stain, one drop) was incubated for 25 min in the dark before imaging. 8 well plates were then used in CO_2 regulated chamber for live cell confocal imaging using a Nikon A1R SIM confocal microscope and 60x oil-immersion objective. The data analysis was performed using the ImageJ (FIJI) software.

***In vitro* Antigen presentation Assay**

Immortalized mouse dendritic cells (DC 2.4) cells were plated in 24 well plates (1×10^5 cells/mL, 500 µL) overnight. The adhered cells were incubated with OVA-mRNA loaded LNPs (1 µg/mL) and OVA-peptide (1 µg/mL) for 24 h. After incubation, cells were washed and stained with Zombie Aqua (1:500) fixable cell viability dye (Biolegend, San Diego, CA) and OVA257-264 (SIINFEKL) peptide bound to H-2Kb antibody, PE-cyanine 7 (0.5 µg/well) to detect the MHC-I class antigen presentation. Cells were analyzed through BD Fortessa flow cytometer. PE-Cy7 channel (for SIINFEKL peptide) was used to determine the percentage of H-2kb SIINFEKL-positive cells.

IVIS imaging mRNA-loaded LNPs

C57BL/6J male mice were intramuscularly injected with Luciferase mRNA containing LNPs (mRNA does 2 µg/mice) or PBS. After 24 h, the mice were intraperitoneally injected with D-luciferin (50 mg/mL in PBS, 200 µL per mouse) and sacrificed after 15 minutes. Draining inguinal lymph nodes and spleens were collected for imaging using the IVIS SpectrumCT In Vivo Imaging System (PerkinElmer). Bioluminescence was measured as average radiance [p/s/cm²/sr].

***In vivo* cellular uptake studies of DID dye-loaded LNPs**

C57BL/6J male mice were intramuscularly injected with 1.5 µg of DiD dye-loaded SM102 and ALC0315 LNPs or PBS as a control. After 24 h, lymph nodes and spleens were harvested from the mice and single-cell suspension samples were prepared from the lymph nodes and spleen. The single-cell suspension was stained for different types of cell markers related to CD45+ immune and CD45- non-immune cells as reported in the Materials section. All samples were measured using the Cytek Aurora flow cytometry instrument and the data was analyzed using Cytobank community software v10.3.

siRNA Functional studies

MDA-MB-231 GFP-expressing cells (2.5×10^5 cells/mL) were seeded in 12 well plates and adhered overnight. The adhered cells were incubated overnight with LNPs containing GFP siRNA or Control siRNA at 60 nM concentration. After incubation, cells were washed with sterile PBS twice and collected into 5 mL flow cytometer tubes. GFP expression was measured using the FITC channel and data was represented as median fluorescent intensity.

Statistical analysis

Data represent the mean ± SD for triplicate measurements in each experiment. The results were evaluated statistically with Graph-Pad Prism 9 software. The significance level is displayed in the figures according to the statistical tests performed such as ANOVA or T-test.

20 mM lipid							
Ionizable LNPs	Molar % of lipids						
	DMG-PEG	ALC0159	DSPC	Cholesterol	SM102	ALC0315	DLin-MC3-DMA
SM102	2	-	23	25	50	-	-
ALC0315	-	2	23	25	-	50	-
DLin-MC3-DMA	2	-	23	25	-	-	50

Table S1. Molar ratios of different lipid components used for the LNP fabrication through the FNP process.

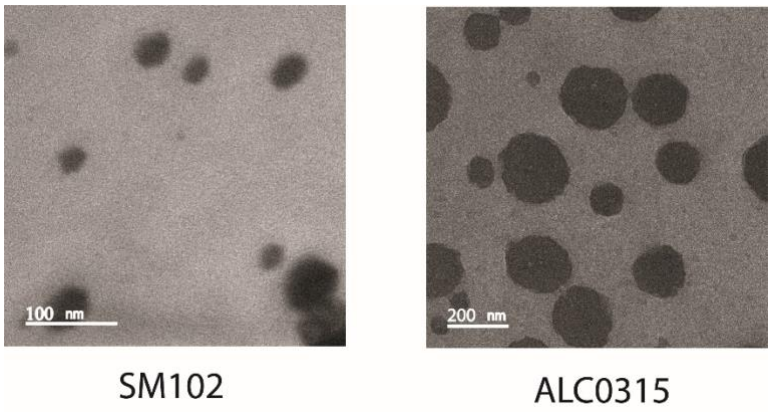


Figure S1. Positively stained TEM images for SM102 and ALC0315 LNPs.

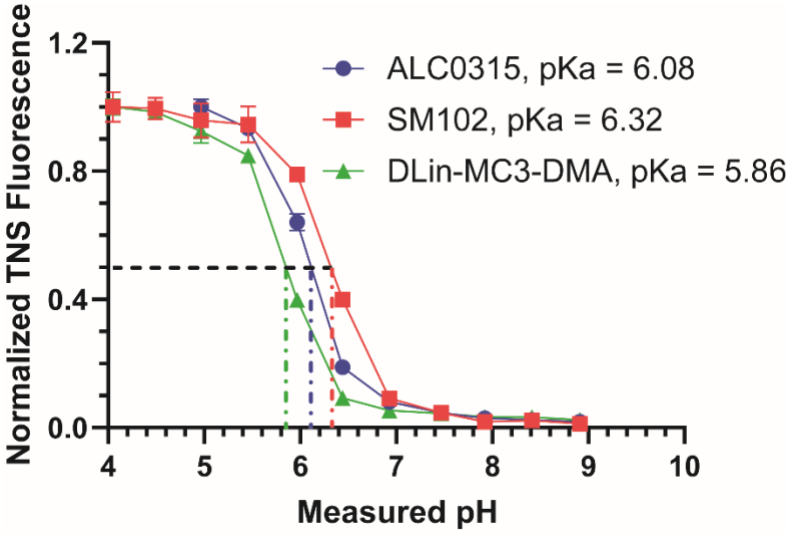


Figure S2. Apparent pK_a of the ionizable LNPs measured by TNS (6-p-Toluidino-2-naphthalenesulfonic acid) assay. The pK_a values of the LNPs were determined by extrapolating the pH value with 50% of the maximum normalized TNS fluorescent intensity. Data represented as mean ± SD (n=3).

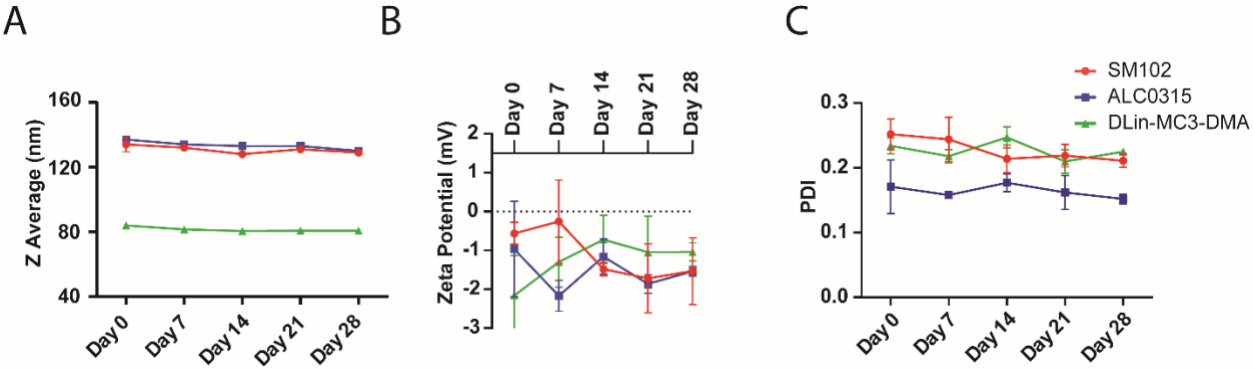
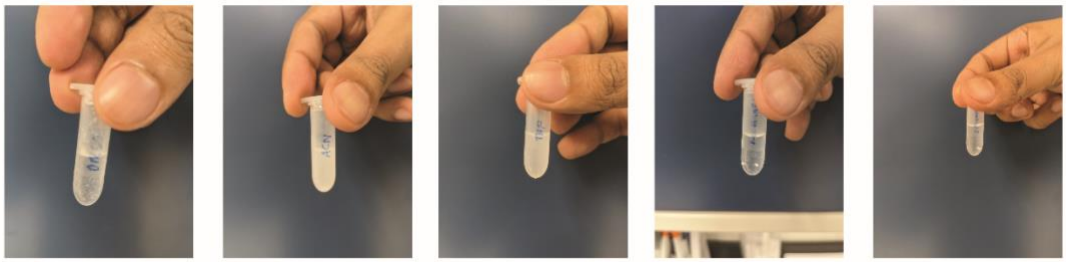


Figure S3. The stability of LNPs prepared through flash nanoprecipitation and stored at 4 °C. A) Particle size of the LNPs was measured over 28 days using dynamic light scattering, and the hydrodynamic diameter is reported as Z average (nm). B) Zeta potential of LNPs was measured over 28 days at different time intervals by electrophoretic light scattering, and the zeta potential is reported as millivolt (mV). C) Polydispersity index (PDI) was measured over 28 days using dynamic light scattering. Data represented as mean ± SD (n=3).

A



DMSO ACN THF Methanol Ethanol

B

LNP formation using Methanol			
LNPs	Z Average Mean (nm)	Polydispersity	Zeta Potential (mV)
SM102	84.2 ± 3.41	0.31	-0.32 ± 1.07
ALC0315	108 ± 0.90	0.35	-1.58 ± 1.12

Figure S4. Selection of water-miscible organic solvents for LNP fabrication via Flash nanoprecipitation (FNP). A) Solubility of the lipid mixture was tested in 1 mL of different organic solvents, only methanol and ethanol were able to dissolve the lipid components, making them suitable solvents for FNP. B) Table depicts the particle size, polydispersity index, and zeta potential of LNPs fabricated using methanol as the organic solvent. Data represented as mean ± SD (n=3).

Ethanol Injection				
	Particle Size [Z average (nm)]	Polydispersity index (PDI)	Zeta Potential (mV)	Encapsulation efficiency (%)
SM102	129 ± 1.58	0.183	-2.48 ± 1.74	88.82 ± 4.58
ALC0315	144 ± 2.07	0.143	-3.10 ± 0.56	85.41 ± 3.25
Dlin-MC3-DMA	104 ± 1.43	0.162	-2.76 ± 0.58	97.51± 5.49

Flash Nanoprecipitation				
	Particle Size [Z average (nm)]	PDI	Zeta Potential (mV)	Encapsulation efficiency (%)
SM102	134 ± 4.68	0.252	-0.56 ± 0.28	96.85 ± 7.13
ALC0315	137 ± 2.11	0.171	-0.96 ± 1.23	97.41 ± 4.27
Dlin-MC3-DMA	133 ± 1.97	0.166	-1.23 ± 0.27	99.50 ± 2.97

Table S2. Comparison of particle size, polydispersity index (PDI), zeta potential, and encapsulation efficiency of SM102, ALC0315, and DLin-MC3-DMA LNPs prepared through ethanol injection and flash nanoprecipitation method. mRNA was used for formulations with SM102 and ALC0315 ionizable LNPs and siRNA was used for DLin-MC3-DMA ionizable LNPs.

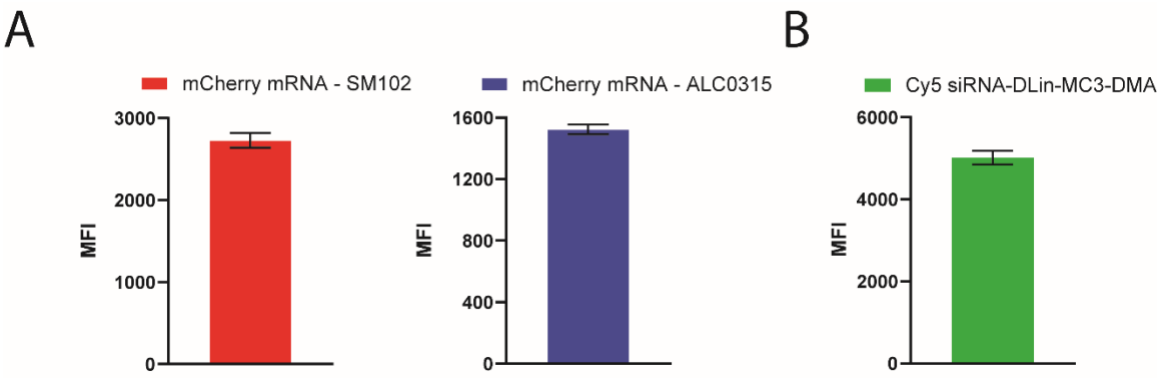


Figure S5. Flow cytometry analysis of A) mCherry mRNA SM102 or ALC0315 LNPs and B) Cy5 siRNA DLin-MC3-DMA LNPs in RAW 264.7 cells. MFI was calculated following 24 h incubation of LNPs with RAW 264.7 cells. Data represented as mean ± SD (n=4).

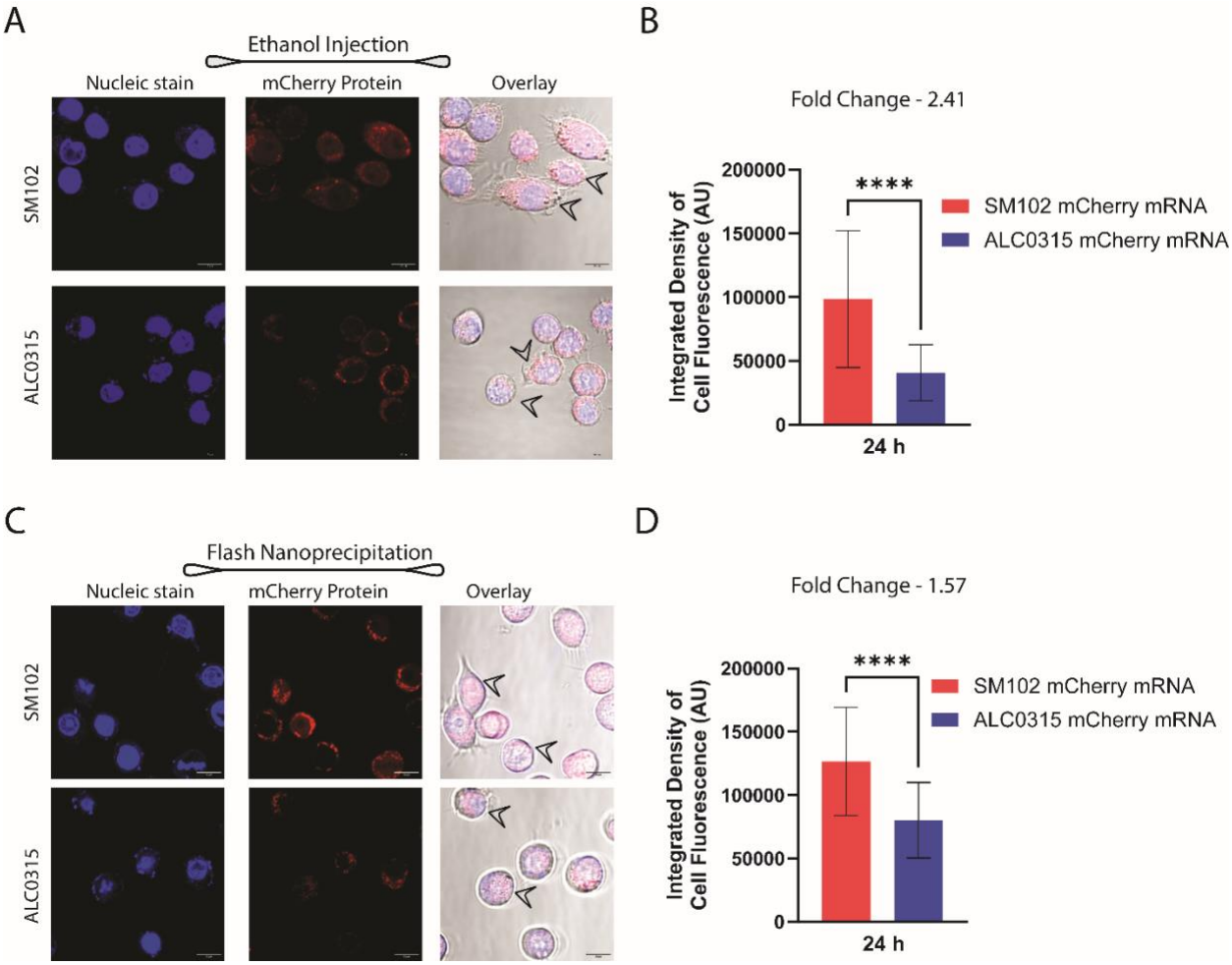


Figure S6. Comparison of LNPs prepared using ethanol injection and FNP methods for intracellular delivery of mRNA in RAW 264.7 cells. SM102 and ALC0315 LNPs prepared through ethanol injection and flash nanoprecipitation methods, were encapsulated with mCherry mRNA and incubated for 24 h with RAW 264.7 cells. mCherry mRNA encoded into mCherry protein (red color) indicates successful cytoplasmic delivery of mRNA by LNPs for both A) ethanol injection formulations and B) Integrated density of fluorescent signal for randomly selected cells that were treated with mCherry mRNA loaded LNPs prepared through ethanol injection method. Data was obtained from minimum 25 cells using 3 separate experiments followed by ImageJ Software mediated analysis. Cells were analyzed after removing the background from non-treated controls. Statistical analysis was performed using unpaired t test with Welch's correction, **** p <0.0001. C) Live cell confocal images of mCherry mRNA loaded LNPs prepared through FNP method. D) Integrated density of fluorescent signals obtained from cells treated with flash nanoprecipitation fabricated LNPs. Statistical analysis was performed using unpaired t test with Welch's correction, **** p <0.0001. Live-cell images were taken using a confocal microscope with a 60X objective and red punctae inside the cells indicated by black arrows. The scale bar is 10 µm.

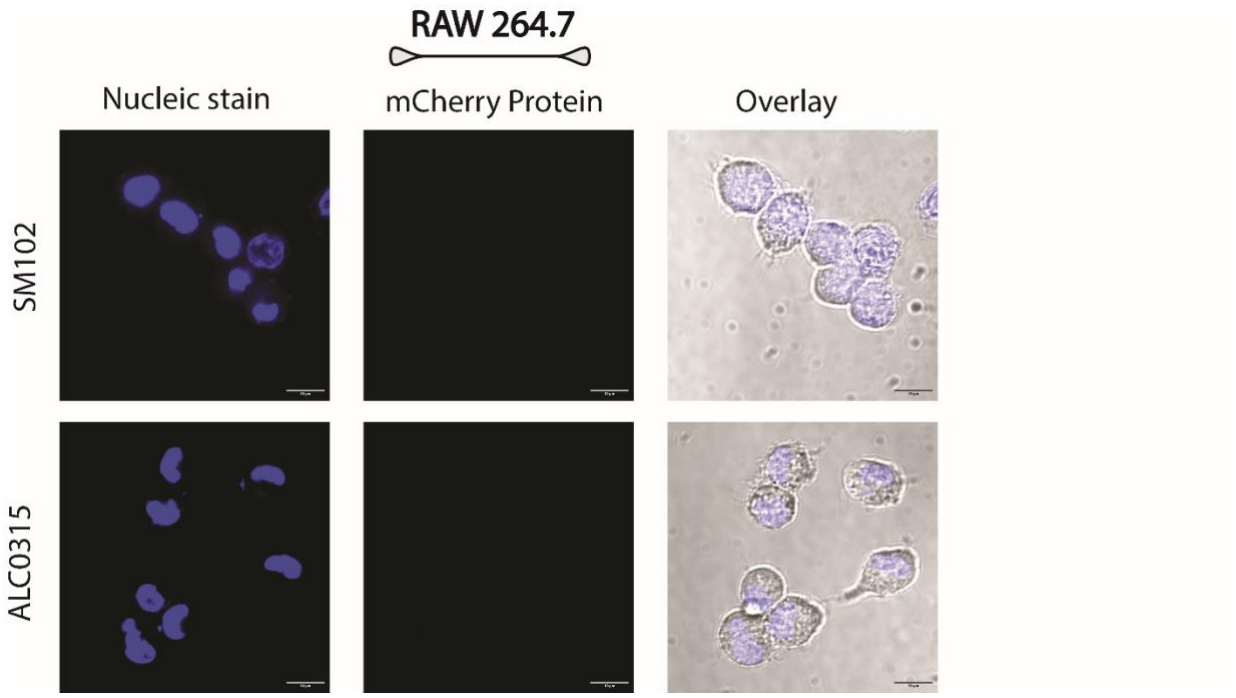


Figure S7. The mRNA functional activity of LNPs prepared through the FNP process and stored at 4 °C. 4 weeks old SM102 and ALC0315 LNP samples encapsulated with mCherry mRNA and incubated for 24 h with RAW 264.7 cells. No translation of mCherry mRNA into mCherry protein (red color) was observed indicating a loss of mRNA functional activity. Live-cell images were taken using a confocal microscope with a 60X objective. The scale bar is 10 µm.

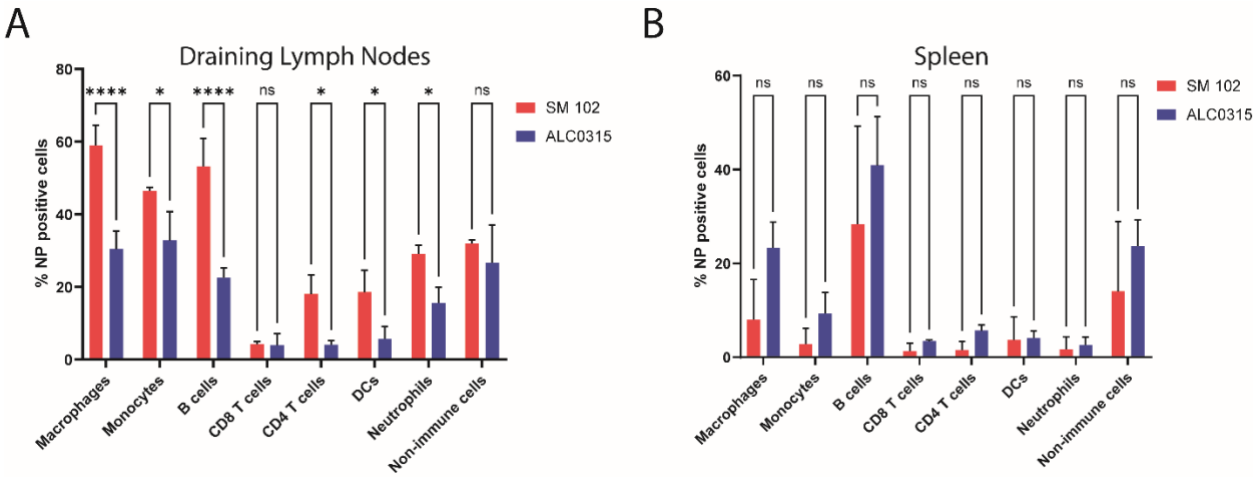


Figure S8. *In vivo* cellular uptake studies of DiD dye-loaded lipid nanoparticles in C57BL/6 mice. Percentage of the DiD nanoparticle positive cells in A) Draining lymph nodes and B) Spleen. The percentage of the cell uptake was measured through flow cytometry. Data represented as mean ± SD (n=3). Significant differences between each timepoint were determined by two-way ANOVA with Šídák's multiple comparison test, $^{****}p < 0.0001$ for macrophages and B cells, $^{*}p = 0.0174$ for monocytes $^{*}p = 0.0148$ for CD4 T cells, $^{*}p = 0.0287$ for DCs, $^{*}p = 0.0206$ for neutrophils.

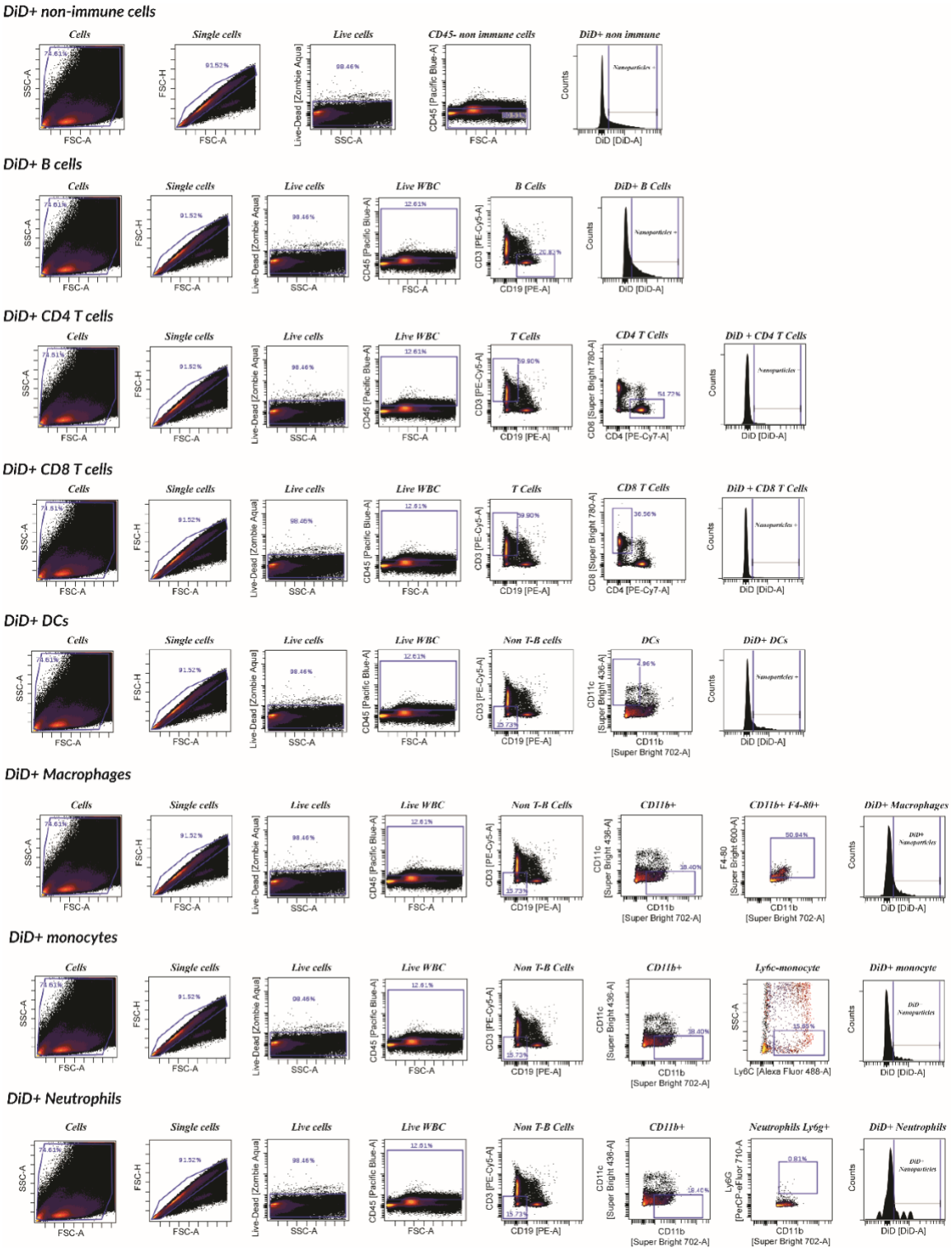


Figure S9. Flow cytometry gating strategy for the analysis of DiD dye-loaded LNPs immune cell uptake. DiD-loaded SM102 and ALC0315 LNPs were intramuscularly injected into C57BL/6 mice. After 24 h of injection, the lymph nodes and spleen were harvested for the immune cell uptake assay. The cells analyzed were non-immune cells, B cells, CD4 and CD8 T cells, dendritic cells (DCs), Macrophages, Monocytes, and Neutrophils.

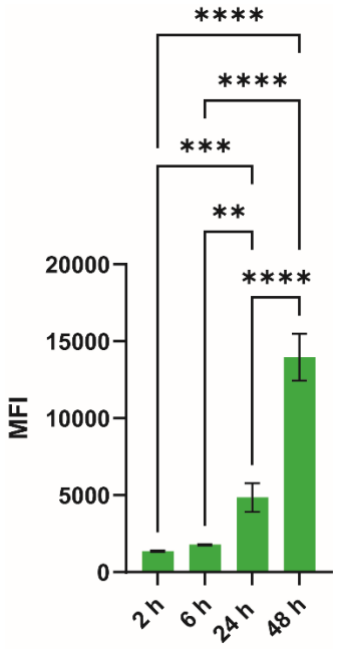


Figure S10. Time-dependent cellular uptake of DiD dye-loaded DLin-MC3-DMA LNPs by MDA-MB-231/Luc cells. After 2, 6, 24, and 48 h of incubation of DiD-loaded LNPs with cells, cellular uptake was measured using flow cytometry and is represented as Median Fluorescent intensity (MFI). Data represented as mean ± SD (n=4). Significant differences between each timepoint were determined by ordinary one-way ANOVA with Tukey's multiple comparison test, ** $p = 0.0019$, *** $p = 0.0007$, and **** $p < 0.0001$.

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