SUPPORTING INFORMATION

Lipid Nanoparticle Spherical Nucleic Acids for Intracellular DNA and RNA Delivery

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1. Materials and Methods

Materials

DNA was synthesized using automated solid support phosphoramidite synthesis (model: MM12, BioAutomation, Inc.). Sequences were purified by reverse phase high-pressure liquid chromatography (HPLC, Agilent Technologies) and characterized using matrix assisted laser desorption ionization-time of flight (MALDI-ToF, Bruker Autoflex III). The DNA sequences used for experiments are listed in **Table S1**. Firefly luciferase mRNA was purchased from TriLink BioTechnologies.

DLin-MC3-DMA was purchased from MedChemExpress. DMPE-PEG(2000) Maleimide, DPPE-PEG(2000) Maleimide, and DSPE-PEG(2000) Maleimide were purchased from Nanocs, Inc. Cholesterol and Triton™-X-100 were purchased from Sigma. DOPC, DSPC, 18:1 DAP, and DOPE were purchased from Avanti Polar lipids, Inc. Lipofectamine™ 2000, Quant-iT™ PicoGreen™ dsDNA reagent, Quant-iT™ RiboGreen™ reagent, and 20× TE buffer were purchased from ThermoFisher. D-Luciferin was purchased from Gold Biotechnologies, and Luc mRNA was purchased from TriLink Biotechnologies.

LNP-SNA Formulation

LNPs were formulated using the ethanol dilution method. Briefly, lipids and cholesterol were dissolved in 100% ethanol. dsDNA was dissolved in 10 mM citrate at pH 4.0 at a mass ratio of 5:1 ionizable lipid:dsDNA. After making both solutions, DNA was rapidly pipette mixed with the ethanol solution at a volume ratio of 3:1. After mixing, LNPs were dialyzed two times in a Pierce™ 3K MWCO microdialysis plate (ThermoFisher) for 60 min against 1× PBS. Then, LNPs were added to microcentrifuge tubes containing 1 equivalent of lyophilized thiol-terminated DNA sequences and shaken at 700 rpm at room temperature overnight to facilitate the reaction of maleimide-functionalized PEG lipids with sulfhydryl-terminated DNA.

LNP-SNA Characterization

LNP-SNAs size and nanoparticle concentration were determined by nanoparticle tracking analysis (NTA) using a Malvern NanoSight NS300 fitted with a NanoSight sample assistant. Nanoparticles were diluted 1:1000 in water and run through the microfluidics at 50 μL/min. Size was determined using

the NTA software with a manually set detection threshold to avoid background. Encapsulation efficiency of dsDNA and RNA was determined by modified Quant-iT™ PicoGreen™ and Quant-iT™ RiboGreen™ (Invitrogen) assays, respectively. Briefly, two separate standard curves were created with the encapsulated nucleic acid. One was in 1× TE Buffer while the other contained 1× TE Buffer supplemented with 0.1% Triton™-X-100. Two samples were created from each nanoparticle, one diluted in TE and one diluted in TE with 0.1% Triton™-X-100. Following, 100 µL of 1× PicoGreen™ (dsDNA) or RiboGreen™ was added on top of the standards and samples and fluorescence of each sample was measured using a plate reader. Concentration of free nucleic acids were determined from the TE standard curve and concentration of total nucleic acids was determined by the particles lysed in 0.1% Triton™-X-100. From this, the encapsulation efficiency was calculated from the following formula: $(TritonX - [TE])/([TritonX])$ or $([Total] - [Free])/([Total])$.

Cellular assays to measure cGAS-STING pathway activation

The Raw 264.7- Lucia™ ISG cell line was purchased from Invivogen. For *in vitro* experiments, Zeocin™, Normocin™, and QUANTI-Luc™ were purchased from Invivogen. All cell lines were cultured according to the manufacturer's specifications. All cell lines were tested for *Mycoplasma* contamination and grown in a humidified atmosphere with 5% CO_2 at 37 °C.

The specified nanoparticle formulations and controls were diluted in Opti-MEM (Gibco) and plated in triplicate in a 96-well plate. Cell were then plated on top of the nanoparticle treatments at 100,000 cells per well. After a 24 h incubation, 20 µL of the media was removed and IRF3 induction was quantified using the Quanti-Luc™ reagent (Invivogen) according to the manufacturer's protocol. To normalize the number of viable cells to the amount of IRF3 induction we achieved, we used the PrestoBlue™ HS cell permeable viability reagent (Thermo Fisher). Next, additional media was removed such that the volume within the plate was 90 µL. 10 µL of PrestoBlue™ was added per well and the plates were incubated for 15 min, at which point the fluorescence was read according to the manufacturer's protocol. The IRF3 induction (luminescence) was then normalized to viable cells (PrestoBlue™ fluorescence) on a well-by-well basis.

LNP-SNAs delivering siRNA in cellular assays

U87-Luc2 cell lines were obtained from ATCC and cultured according to the manufacturer's specifications. To assess siRNA-mediated gene silencing, the top five LNP-SNA candidates from the cGAS-STING pathways screening were formulated with siLuc2 and paired with control LNP-SNAs formulated with siGFP. Therefore, gene silencing could be read out as a decrease in luminescence due to silencing of Luc2.

The specified nanoparticle formulations and transfected siRNA controls were diluted in Opti-MEM (Gibco) and plated in triplicate in a 96-well plate. Cells were then plated on top of the nanoparticle treatments at 50,000 cells per well. After a 24 h incubation, 120 μL of the media was removed and 20 μL of CellTiter-Fluor™ reagent (Promega) was added to measure the number of viable cells within each well. After a 30 min incubation at 37 °C, fluorescence was read according to the manufacturer's protocol. Wells were subsequently washed with 100 μL of PBS three times. Luc2 luminescence was read using the Luciferase Assay System (Promega). Luc2 gene silencing was assessed in arbitrary units normalized to the CellTiter-Fluor™ viability.

Animal handling

Female mice (C57Bl/6) in the age range of 8-12 weeks were obtained from The Jackson Laboratory and maintained in conventional housing. All animals used were handled according to methods and procedures approved by the Institutional Animal Care and Use Committee at Northwestern University.

Luciferase (Luc2) mRNA expression

Cleancap® Luciferase mRNA was purchased from TriLink Biotechnologies. Mice were given a single bolus injection of 0.1 mg kg^{-1} of mRNA-containing formulations. After 6 h, mice were injected intraperitoneally with 150 mg kg^{-1} of D-luciferin. Animals were then sacrificed, and major organs were harvested and soaked in a 300 μg mL^{-1} solution of D-luciferin. Individual organs were then imaged using an IVIS Spectrum instrument (Perkin Elmer).

Statistical analysis

Design of Experiment (DoE) was used to create library designs A and B. DoE and statistical analysis were performed using R version 3.6.1 (cran.r-project.org) and JMP Pro 15. Descriptions of the DoE and statistical analysis are available in greater detail in section 4 of the Supporting Information. Statistical significance is defined as having a p-value of less than 0.05 in this study. All results are expressed as the mean ± SE and number of biological replicates (n) as noted in the figure captions. To design the 1st generation library, we used a definitive screening design to estimate the main and two-factor effects using less experimental runs. The factors used in Library A were: lipid-PEG Length, PEG mol %, chol. mol %, and ionizable lipid. For Library B, we used JMP to design a $3^3 2^2$ resolution IV fractional factorial design. The three-level factors used in Library B were phospholipid, PEG mol %, and chol mol %. The two-level factors used were PEG length and DNA sequence. Again, in this design, we were only interested in the main effects and first order interaction effects. Using a Custom Experiment Design on JMP, the minimum number of nanoparticles to run was 37 of the possible of the 108 full factorials. JMP software was used to plot the main effects and first order interaction effects in this experiment. To calculate the least squares regression model, we reduced the model until only significant main effects and first order interaction effects were included.

For mouse experiments, the free PS Power and Sample Size calculation tool Version 3.6.1 (Vanderbilt) was used to determine the minimum sample size for which the statistical power was greater than 0.8. This was generally 5-6 mice per group for Luc mRNA delivery experiment.

2. Synthesis and Characterization of LSNAs

Table S1. LNP-SNA formulations used

Lib_#	Phospholipid	ionizable lipid	lipid-PEG	surface DNA	% phospholipid	% chol.	% ionizable lipid	% lipid-PEG	EE (%)	size (nm)	sd (nm)
A_1	DOPC	DLin-MC3	C18	T21	10	35	50	5	82	159	79
A_2	DOPC	18:1 DAP	C14	T21	24	25	50	1	84	279	107
A_3	DOPC	DLin-MC3	C18	T21	20	25	50	5	67	227	81
A_4	DOPC	18:1 DAP	C14	T21	14	35	50	1	85	221	105
A_5	DOPC	18:1 DAP	C18	T21	12.5	35	50	2.5	86	203	85
A_6	DOPC	DLin-MC3	C14	T21	22.5	25	50	2.5	74	246	91
A_7	DOPC	DLin-MC3	C16	T21	12.5	35	50	2.5	78	195	87
A_8	DOPC	18:1 DAP	C16	T21	22.5	25	50	2.5	83	190	91
A_9	DOPC	DLin-MC3	C14	T21	10	35	50	5	56	236	71
A_10	DOPC	18:1 DAP	C18	T21	24	25	50	1	88	277	91
A_11	DOPC	DLin-MC3	C16	T21	22.5	25	50	2.5	71	222	71
A_12	DOPC	18:1 DAP	C16	T21	12.5	35	50	2.5	87	178	83
A_13	DOPC	18:1 DAP	C16	T21	20	25	50	5	85	231	81
A_14	DOPC	DLin-MC3	C16	T21	14	35	50	1	79	227	60
A_15	DOPC	18:1 DAP	C16	T21	10	35	50	5	83	187	84
A_16	DOPC	DLin-MC3	C16	T21	24	25	50	1	71	229	69
A_17	DOPC	18:1 DAP	C14	T21	24	25	50	1	83	209	81
A_18	DOPC	DLin-MC3	C18	T21	10	35	50	5	80	144	79
B_1	DSPC	DLin-MC3	C16	GGT7	12.5	35	50	2.5	100	194	80
B_2	DSPC	DLin-MC3	C16	GGT7	12.5	35	50	2.5	103	197	73
B_3	DOPE	DLin-MC3	C14	GGT7	22.5	25	50	2.5	77	220	77
B_4	DOPE	DLin-MC3	C14	GGT7	22.5	25	50	2.5	84	217	76
B_5	DOPC	DLin-MC3	C16	T21	1.5	45	50	3.5	95	202	73
B_6	DOPC	DLin-MC3	C16	T21	1.5	45	50	3.5	97	184	76
B_7	DOPE	DLin-MC3	C16	GGT7	21.5	25	50	3.5	94	194	66
B_8	DSPC	DLin-MC3	C14	T21	22.5	25	50	2.5	93	274	82
B_9	DOPE	DLin-MC3	C16	GGT7	13.5	35	50	1.5	98	218	78
B_10	DSPC	DLin-MC3	C16	GGT7	23.5	25	50	1.5	101	317	94
B_11	DOPE	DLin-MC3	C14	T21	12.5	35	50	2.5	86	232	75
B_12	DOPC	DLin-MC3	C14	GGT7	1.5	45	50	3.5	91	233	79
B_13	DOPE	DLin-MC3	C16	T21	3.5	45	50	1.5	98	194	71
B_14	DOPC	DLin-MC3	C16	GGT7	22.5	25	50	2.5	99	225	82
B_15	DSPC	DLin-MC3	C14	T21	3.5	45	50	1.5	92	239	82
B_16	DSPC	DLin-MC3	C14	T21	11.5	35	50	3.5	93	242	77
B_17	DSPC	DLin-MC3	C14	GGT7	2.5	45	50	2.5	75	276	70
B_18	DOPE	DLin-MC3	C14	T21	23.5	25	50	1.5	72	303	70
B_19	DOPE	DLin-MC3	C14	GGT7	3.5	45	50	1.5	73	229	93
B_20	DOPC	DLin-MC3	C14	GGT7	12.5	35	50	2.5	76	231	89
B_21	DOPC	DLin-MC3	C16	GGT7	11.5	35	50	3.5	79	210	76
B_22	DSPC	DLin-MC3	C16	T21	2.5	45	50	2.5	79	229	79
B_23	DOPC	DLin-MC3	C14	T21	21.5	25	50	3.5	75	220	76
B_24	DSPC	DLin-MC3	C14	GGT7	13.5	35	50	1.5	80	239	81
B_25	DOPC	DLin-MC3	C16	T21	23.5	25	50	1.5	80	240	95
B_26	DSPC	DLin-MC3	C16	GGT7	1.5	45	50	3.5	76	237	83
B_27	DOPE	DLin-MC3	C14	T21	1.5	45	50	3.5	74	266	91
B_28	DSPC	DLin-MC3	C16	T21	11.5	35	50	3.5	72	243	83
B_29	DOPE	DLin-MC3	C14	GGT7	11.5	35	50	3.5	72	270	84
B_30	DOPC	DLin-MC3	C14	T21	2.5	45	50	2.5	76	295	84
B_31	DOPE	DLin-MC3	C16	T21	22.5	25	50	2.5	74	223	79
B_32	DOPC	DLin-MC3	C14	T21	13.5	35	50	1.5	77	277	90
B_33	DOPC	DLin-MC3	C16	GGT7	3.5	45	50	1.5	79	187	72
B_34	DOPE	DLin-MC3	C16	GGT7	2.5	45	50	2.5	76	214	81
B_35	DSPC	DLin-MC3	C14	GGT7	21.5	25	50	3.5	79	206	72
B_36	DOPC	DLin-MC3	C16	T21	12.5	35	50	2.5	80	232	73
B_37	DOPC	DLin-MC3	C14	GGT7	23.5	25	50	1.5	81	203	76
C_1	DOPC	DLin-MC3	C16	GGT7	2.5	45	50	2.5	85	212	88
C_2	DOPC	DLin-MC3	C16	GGT7	1.5	45	50	3.5	90	167	106
C_3	DSPC	DLin-MC3	C16	GGT7	3.5	45	50	1.5	86	147	99
C_4	DOPE	DLin-MC3	C16	GGT7	3.5	45	50	1.5	87	102	62

Table S2. DNA sequences used

Formulation	DNA Sequence (5'-3')
T21 SNA	TTTTTTTTTTTTTTTTTTTT -SH[a]
(GGT)7 SNA	GGT GGT GGT GGT GGT GGT GGT GGT-SH[a]

[a] Synthesized using Dithiol Serinol CpG (Glen Research), subsequently reduced using 100 mM DTT in 20 mM Tris HCl pH 8.3-8.5 for 30 min and desalted on a NAP-10 column (Cytiva).

Figure S1. Agarose gel to confirm surface DNA conjugation
A.) 1% agarose gel run in 1X TAE buffer at 85 V for 45 min. to confirm conjugation of T21 DNA to LNPs after 2 h shaking at RT. One equivalent of a T21-SH DNA sequence labeled with Cy5.5 was added to formulation B-35, which contains 3.5% C14-PEG(2000)-Maleimides. Presence of bands at higher MW than free Cy5.5 DNA (Lane 1), indicates that they are conjugated to the lipid-PEG.

3. Screening LNP-SNA Structures

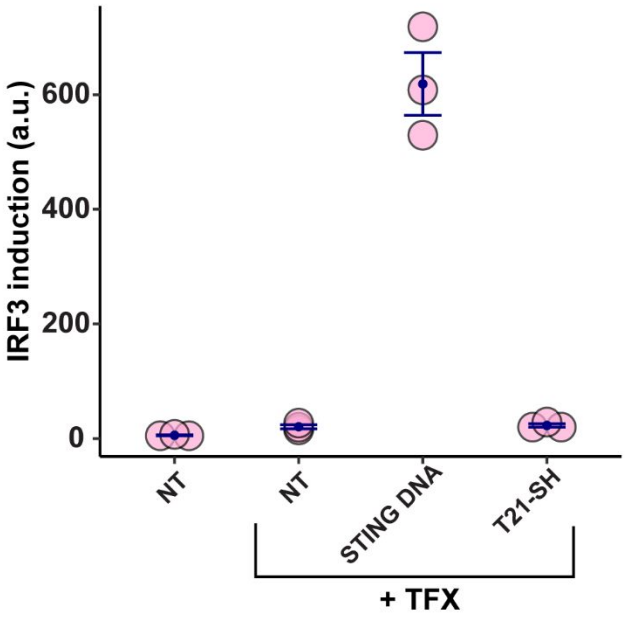

Figure S2. DNA sequence controls for Library A screening.
IRF3 induction of DNA sequences used in Library A measured after 24 h. Sequences were transfected with Lipofectamine 2000™ according to manufacturer's protocol. (TFX = with transfection; NT = not treated.)

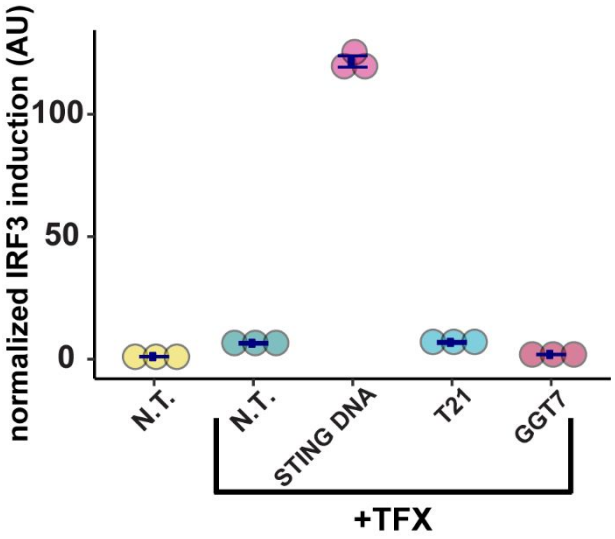

Figure S3. Sequence controls used in Library B screening
IRF3 induction of DNA sequences used in Library B measured after 24 h. Sequences were transfected with Lipofectamine 2000™ according to manufacturer's protocol. (TFX = with transfection; NT = not treated.)

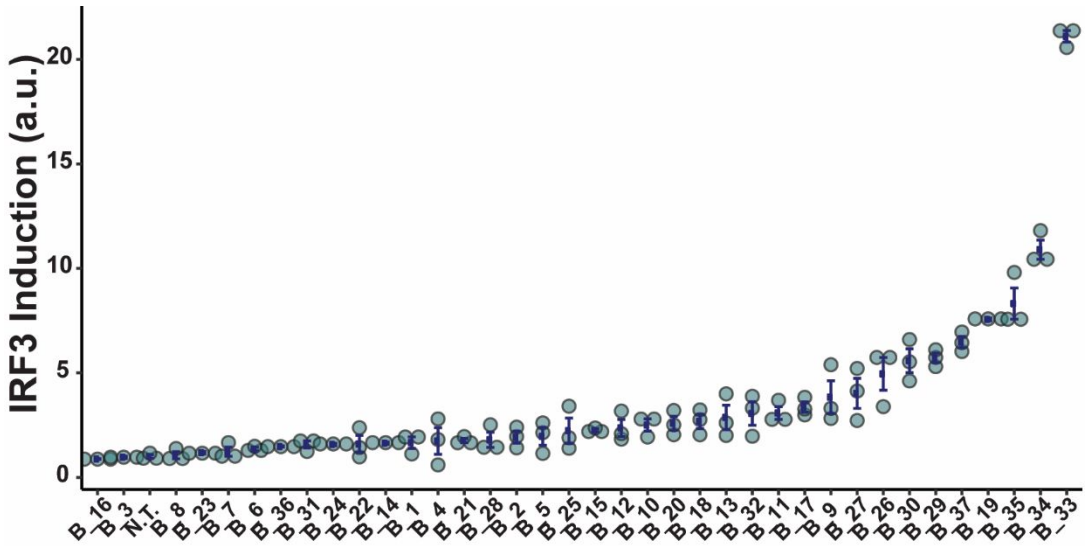

Figure S4. Results of Library B screening
IRF3 induction of LNP-SNAs used in Library B measured after 24 h. Results normalized to untreated wells. (NT = not treated, n = 3 biologically independent replicates)

Table S3. Predictor Rank in Library B using Bootstrap Forest

Predictor	Portion		Rank
chol%	0.3539		1
DNA_seq	0.2422		2
PEG%	0.1902		3
Phospholipid	0.1563		4
Peg length	0.0574		5

Table S4. Summary of linear model used in Lib. B Analysis

Summary of Fit	
RSquare	0.998965
RSquare Adj	0.987577
Root Mean Square Error	0.088216
Mean of Response	0.749427
Observations (or Sum Wgts)	37

Analysis of Variance				
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	33	22.527795	0.682660	87.7217
Error	3	0.023346	0.007782	**Prob > F**
C. Total	36	22.551142		0.0017*

Effect Tests					
Source	**Nparm**	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Phospholipid	2	2	1.0969107	70.4764	0.0030*
PEG%	2	2	0.6603282	42.4260	0.0063*
chol%	2	2	4.7502410	305.2025	0.0003*
Peg length	1	1	0.0899112	11.5536	0.0425*
DNA_seq	1	1	1.6965052	218.0006	0.0007*
Phospholipid*PEG%	4	4	5.0465053	162.1187	0.0008*
Phospholipid*chol%	4	4	2.0945087	67.2860	0.0029*
PEG%*chol%	4	4	2.6670092	85.6775	0.0020*
Phospholipid*Peg length	2	2	0.2712912	17.4304	0.0223*
PEG%*Peg length	2	2	0.4875167	31.3229	0.0098*
chol%*Peg length	2	2	0.6619163	42.5281	0.0063*
Phospholipid*DNA_seq	2	2	0.5468453	35.1348	0.0083*
PEG%*DNA_seq	2	2	0.5789667	37.1986	0.0076*
chol%*DNA_seq	2	2	1.3582040	87.2645	0.0022*
Peg length*DNA_seq	1	1	0.1130788	14.5306	0.0317*

Source	LogWorth		PValue
chol%	3.466		0.00034
DNA_seq	3.171		0.00067
Phospholipid*PEG%	3.108		0.00078
PEG%*chol%	2.697		0.00201
chol%*DNA_seq	2.658		0.00220
Phospholipid*chol%	2.541		0.00287
Phospholipid	2.522		0.00301
chol%*Peg length	2.201		0.00629
PEG%	2.200		0.00631
PEG%*DNA_seq	2.117		0.00763
Phospholipid*DNA_seq	2.082		0.00829
PEG%*Peg length	2.010		0.00977
Phospholipid*Peg length	1.652		0.02230
Peg length*DNA_seq	1.498		0.03175
Peg length	1.372		0.04249

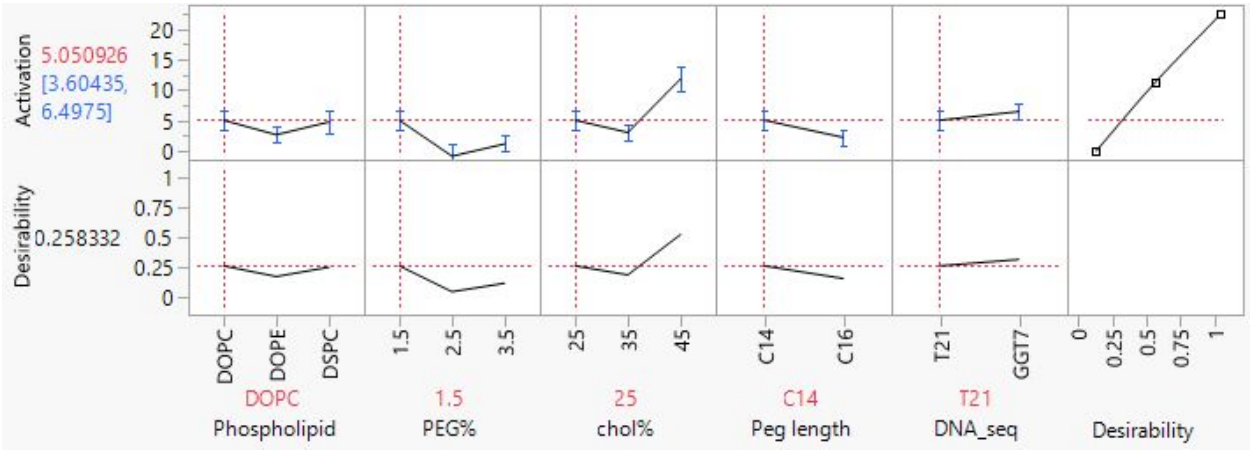

Figure S5. Example main effects and desirability plots for SNAs in Library B.
Activation (top row) and desirability (bottom row) of LNP-SNAs in Library B as a function of formulation parameters phospholipid, PEG%, cholesterol %, PEG length, and DNA sequence. The plot demonstrates how levels of each factor predict the activation or desirability (highest output of the Library scaled to 1, lowest output scaled to 0). In JMP, the red dashed lines can be moved to change levels of each factor, and the predicted activation is shown in red, along with a 95% confidence interval (blue).

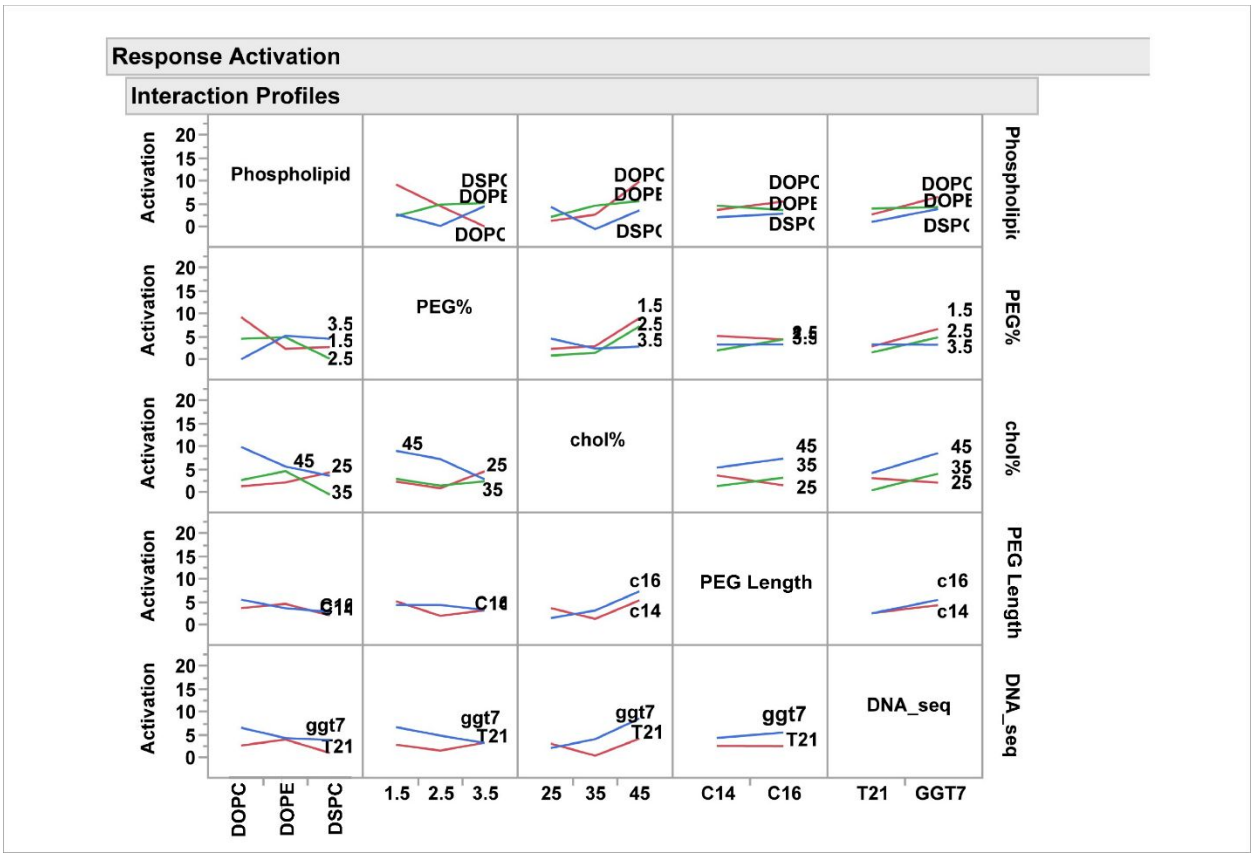

Figure S6. Interaction effects between parameters in library B

Interaction of each factor's levels (bottom) with another factor (right side). Plot reflects activity (a.u.), on the Y axis, as a function of the interaction between two factor levels in Library B.

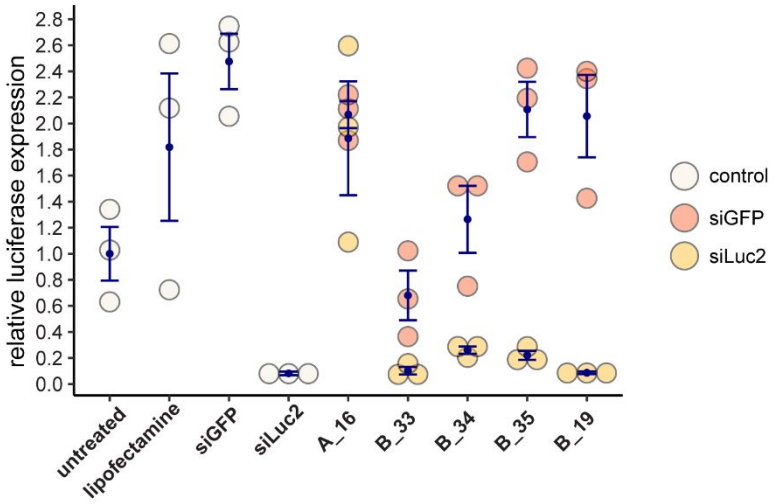

Figure S7. Initial Screening of siRNA-containing LNP-SNAs in B16-F10-Luc2 cells
B16-F10-Luc2 cells were treated with either transfected sequence controls (white) or LNP-SNAs formulated with either a control siGFP (orange) or siLuc2 (yellow) targeting sequence at 100 nM concentration for 24 h. (N.T. = not treated)

Table S5. mRNA encapsulation efficiency of top LNP-SNA candidates

Sample	Encapsulation efficiency (%)	Error (± %)
B-35	46	4
B-34	70	2
B-19	78	3

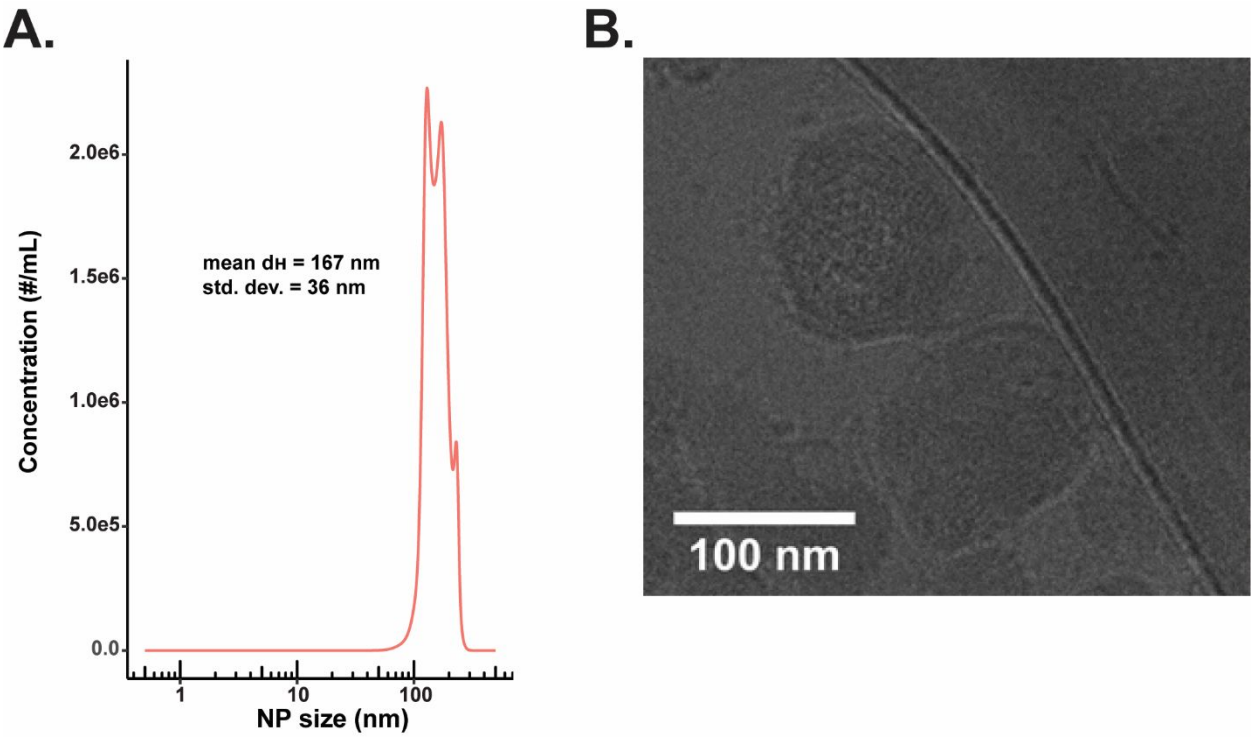

Figure S8. Characterization of LNP-SNA B-19 with encapsulated Luc2 mRNA
(A) Plot of the average of three NanoSight runs of B-19 LNP-SNA. (B) Cryo-TEM image of the same SNA.

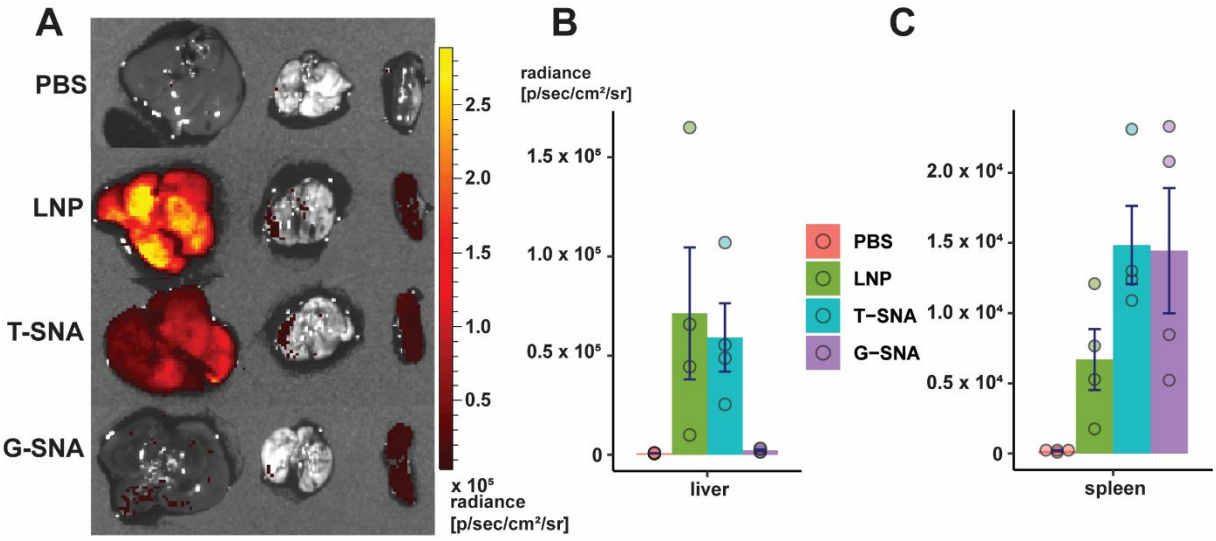

Figure S9. LNP-SNA mRNA expression profile is sequence-dependent.
(A) Luciferase mRNA in liver, lungs, and spleen by treatment. Luminescence was detected in harvested organs 6 h after administration of 0.1 mg kg^{-1} Luc mRNA. (B) LNP and T-SNA exhibit significant liver mRNA expression while G-SNA does not. (C) G-SNA exhibits mRNA expression in the spleen at levels comparable to LNP and T-SNA. (T-SNA is LNP functionalized with T21-SH DNA, G-SNA is LNP functionalized with (GGT)7-SH DNA sequence, N=4 biologically independent replicates).

4. Discussion of DoE Optimization Process

With the initial selection of factors, we had a $2^2\,3^2$ design. Because a full-factorial screening using this design would be 36 different nanoparticles, we used a definitive screening design (Jones and Nachtsheim) to estimate the main and two-factor effects using less experimental runs. In definitive screening experiments, unlike resolution III fractional factorial designs, the main effects are not confounded by two-factor interactions. Additionally, unlike resolution IV designs, two-factor interactions are not completely confounded with other two-factor interactions. These properties make it easier to move directly from screening to optimization. (For detailed descriptions of the creation and analysis of definitive screening and fractional factorial designs, we recommend the reader refer to the text *Design and Analysis of Experiments* (8^{th} ed.) by Douglas C. Montgomery.)

In order to use a definitive screening design, we had to decompose the three level factors into two two-level factors (see **Table S3**). Using JMP software, we designed the nanoparticles required for definitive screening.

Following the use of a definitive screening design, we used a resolution IV fractional factorial design to perform further optimization. The base formulations were derived from the particles that exhibited significant IRF3 induction presented in Figure 2E. It is important to note that we used *coded* units instead of the *natural* or *engineering* units of each component presented in **Table S2**. Coded units make the magnitude of the coefficients in the model directly comparable so that we can compare the relative size of factor effects, e.g., we can directly compare the effect size of changing percent cholesterol or changing percent PEG-lipid without the results being masked by large differences in engineering units.

Standard Least Squares Regression Model

A model describing all of the first and second-order effects was constructed using JMP. The program lists the effects of each parameter and second-order effect in the model as well as the LogWorth ($-\log_{10}$(FDR P-value)).