Supporting Information

An Orthogonal Array Optimization of Lipid-like Nanoparticles for mRNA Delivery in Vivo

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General synthetic procedures and spectral data:

*Synthesis of compound **3***. To a solution of diamine (75 mmol) in $CHCl_3$ (70 mL) was added a solution of Boc_2O (14.4 mmol) in $CHCl_3$ (30 mL) via an additional funnel over 2.5 h. The resulting suspension was stirred and 100 mL of $NaHCO_3$ (1N) was slowly added to form a bi-layer solution. The organic layer was washed with 100 mL of 1N $NaHCO_3$ and 20 mL of brine, and then dried over solid $MgSO_4$ for 2 h. The solution was then filtered, evaporated and dried under high vacuum in order to afford compound **3**. Yield: n=2, 91%; n=3, commercially available reagent; n=4, 63%; n=5, 95%; n=6, commercially available reagent; n=7, 95%; n=8, 86%.

*Synthesis of compound **4**.*[1] To a solution of compound **2** (1.88 mmol) in CH_2Cl_2 (30 mL) was added 10 mL of pyridine and cooled in an ice bath. A solution of compound **3** (7.52 mmol) in CH_2Cl_2 (30 mL) was added dropwise with stirring. The reaction mixture was then allowed to warm to RT, diluted with 100 mL of CH_2Cl_2 and washed twice with 50 mL of water, 50 mL of saturated $NaHCO_3$, and 50 mL of brine. The solution was dried over solid $MgSO_4$ for 2 h and concentrated. The residue was purified by column chromatography using a CombiFlash Rf system with a RediSep Gold Resolution silica column (Teledyne Isco) with gradient elution from 100% CH_2Cl_2 to CH_2Cl_2/MeOH/NH_4OH (75/22/3 by volume) to give compound **4**. Yield: n=2, 74%; n=3, 68%; n=4, 65%; n=5, 69%; n=6, 81%; n=7, 37%; n=8, 61%.

*Synthesis of compound **1***. To a suspension of compound **4** (1.41 mmol) in CH_2Cl_2 (20 mL) was added trifluoroacetic acid (TFA, 1.41 mmol). The mixture was stirred at RT for 1 h and monitored with thin layer chromatography (TLC). Upon completion of the reaction, the solvent was evaporated and the residue was dissolved in MeOH and concentrated. After solidification in EtOAc, compound **1** was dried under an oil pump and afforded in quantitative yield.

^{1}H NMR spectra were recorded at 300 or 400 MHz on the Bruker instrument. ^{1}H NMR chemical shifts were reported as δ values in ppm relative to TMS. Mass spectra were obtained on a Micromass Q-TOF micro Mass Spectrometer.

N^1,*N*^{3},*N*^{5}-tris(2-(didodecylamino)ethyl)benzene-1,3,5-tricarboxamide (**TT2**): yield (82%). ^{1}H NMR (300 MHz, CDCl3, δ) 8.88 (3H, br), 8.56 (3H, s), 3.81 (6H, m), 3.32 (6H, m), 3.04-3.08 (12H, m), 1.69 (12H, s), 1.20-1.30 (108H, m), 0.88 (18H, tri, *J* = 6.9 Hz). MS (*m/z*): $[M + H]^+$ calcd. for $C_{87}H_{169}N_6O_3$, 1346; found, 1346.

N^1,*N*^{3},*N*^{5}-tris(3-(didodecylamino)propyl)benzene-1,3,5-tricarboxamide (**TT3**): yield (50%). ^{1}H NMR (300 MHz, CDCl3, δ) 8.53 (3H, br), 8.40 (3H, s), 3.57 (6H, m),2.67-3.60 (6H, tri, *J* = 5.4 Hz), 2.58-2.48 (12H, tri, *J* = 7.5 Hz), 1.90-1.70 (12H, m), 1.57-1.38 (12H, m), 1.35-1.17 (96H, m), 0.89 (18H, tri, *J* = 6.9 Hz). MS (*m/z*): $[M + H]^+$ calcd. for $C_{90}H_{175}N_6O_3$, 1388; found, 1388.

N^1,*N*^{3},*N*^{5}-tris(4-(didodecylamino)butyl)benzene-1,3,5-tricarboxamide (**TT4**): yield (31%). ^{1}H NMR (300 MHz, CDCl3, δ) 8.33 (3H, s), 7.39 (3H, br), 3.5-3.42 (6H, m),2.65-2.46 (18H, m), 1.75-1.55 (12H, m), 1.52-1.37 (12H, m), 1.35-1.17 (102H, m), 0.90 (18H, tri, *J* = 6.9 Hz). MS (*m/z*): $[M + H]^+$ calcd. for $C_{93}H_{181}N_6O_3$, 1430; found, 1430.

N^1,*N*^{3},*N*^{5}-tris(5-(didodecylamino)pentyl)benzene-1,3,5-tricarboxamide (**TT5**): yield (45%). ^{1}H NMR (300 MHz, CDCl3, δ) 8.40 (3H, s), 6.62 (3H, s), 3.56-3.45 (6H, m),2.47-2.39 (18H, m), 1.76-1.65 (18H, m), 1.52-1.37 (24H, m), 1.35-1.20 (90H, m), 0.89 (18H, tri, *J* = 6.9Hz). MS (*m/z*): $[M + H]^+$ calcd. for $C_{96}H_{187}N_6O_3$, 1472; found, 1472.

N^1,*N*^{3},*N*^{5}-tris(6-(didodecylamino)hexyl)benzene-1,3,5-tricarboxamide (**TT6**): yield (53%). ^{1}H NMR (300 MHz, CDCl3, δ) 8.38 (3H, s), 6.52 (3H, br), 3.52-3.45 (6H, m), 2.47-2.39 (18H, m), 1.72-1.63 (18H, m), 1.54-1.36 (30H, m), 1.32-1.21 (90H, m), 0.90 (18H, tri, *J* = 6.9Hz). MS (*m/z*): [M

+ H]$^+$ calcd. for $C_{99}H_{193}N_6O_3$, 1515; found, 1515.

N^1,N^3,N^5-tris(7-(didodecylamino)heptyl)benzene-1,3,5-tricarboxamide (**TT7**): yield (75%). ^{1}H NMR (300 MHz, CDCl3, δ) 8.40 (3H, s), 6.72 (3H, s), 3.48 (6H, m), 2.50 (18H, m), 1.70-1.56 (6H, m), 1.55-1.34 (30H, m), 1.37-1.27 (108H, m), 0.89 (18H, tri, *J* = 6.9 Hz). MS (*m/z*): [M + H]$^+$ calcd. for $C_{102}H_{199}N_6O_3$, 1557; found, 1557.

N^1,N^3,N^5-tris(8-(didodecylamino)octyl)benzene-1,3,5-tricarboxamide (**TT8**): yield (quantitative). ^{1}H NMR (300 MHz, CDCl3, δ) 8.40 (3H, s), 6.86 (3H, br), 3.47 (6H, m), 2.82-2.52 (18H, m), 1.73-1.42 (30H, m), 1.42-1.17 (120H, m), 0.90 (18H, tri, *J* = 6.9 Hz). MS (*m/z*): [M + H]$^+$ calcd. for $C_{105}H_{205}N_6O_3$, 1599; found, 1599.

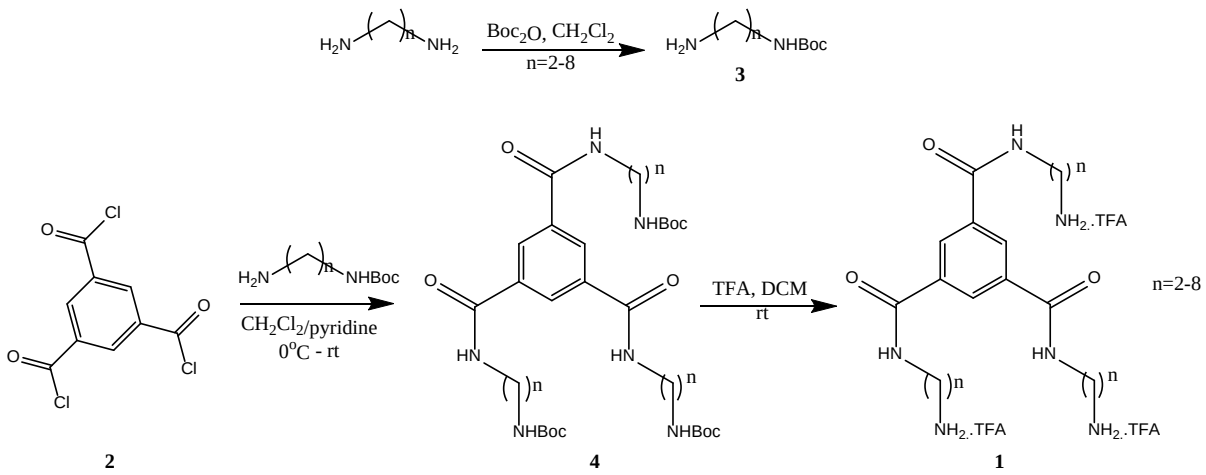

Figure S1. The synthetic route to compound **1**. Benzene-1,3,5-tricarbonyl trichloride (**2**) was reacted with Boc-protected diamine (**3**) to produce the intermediates (**4**). Deprotection of **4** gave compound **1**.

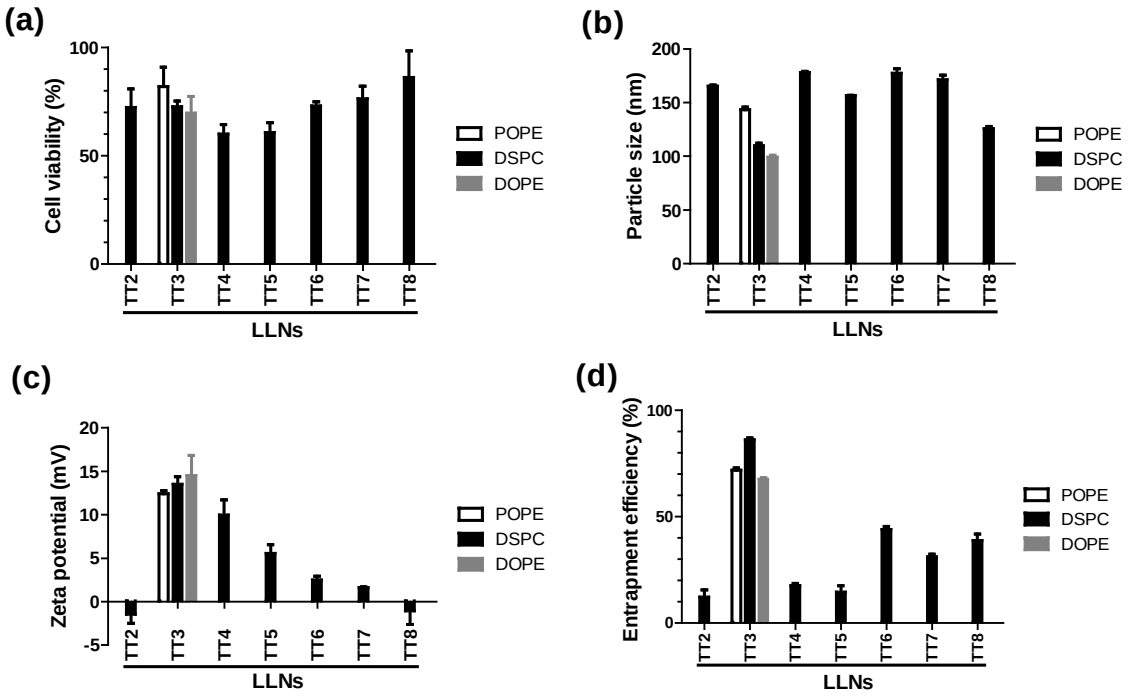

Figure S2. The cell viability (a), particle size (b), zeta potential (c), and entrapment efficiency (d) of LLNs.

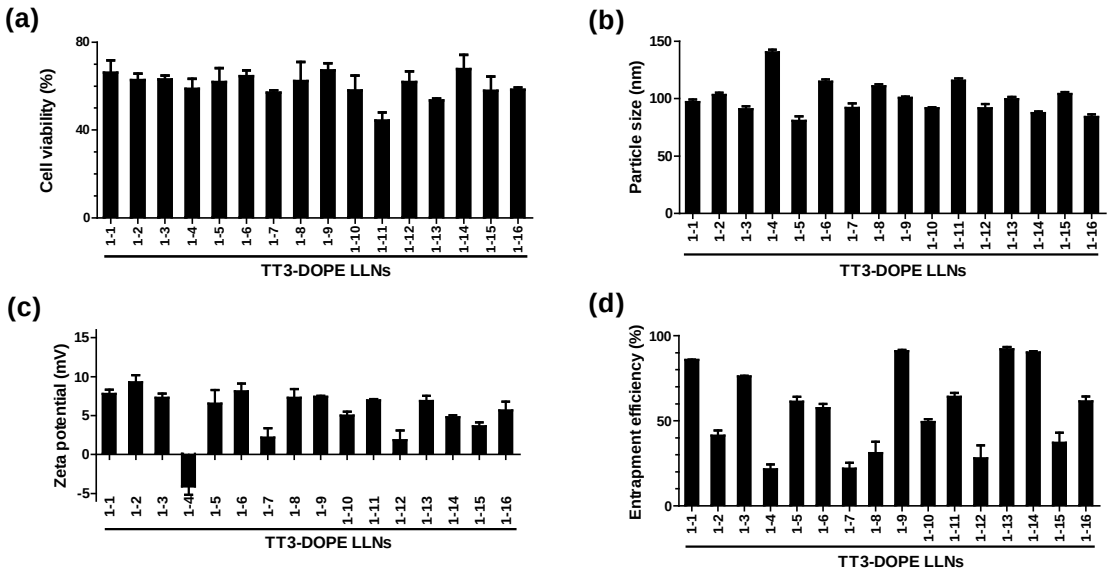

Figure S3. The cell viability (a), particle size (b), zeta potential (c), and entrapment efficiency (d) of **TT3**-DOPE LLNs formulated through the first orthogonal array.

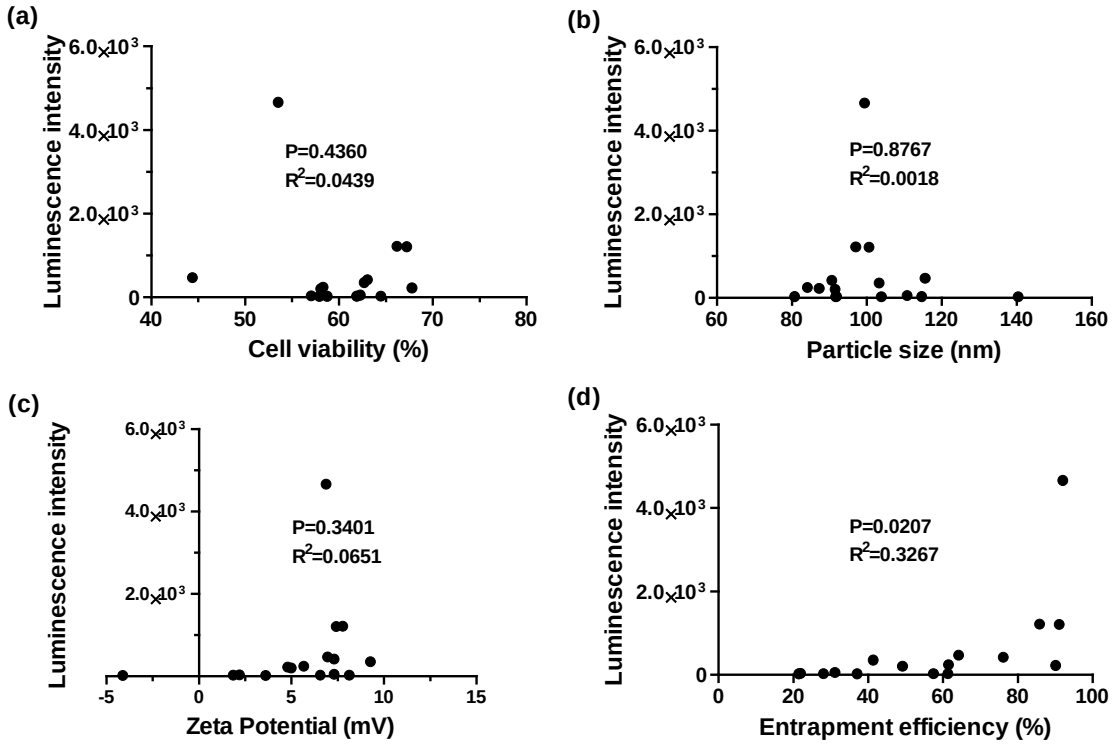

Figure S4. The correlation between transfection efficiency of **TT3**-DOPE LLNs formulated through the first orthogonal array and corresponding cell viability, particle size, zeta potential, and entrapment efficiency.

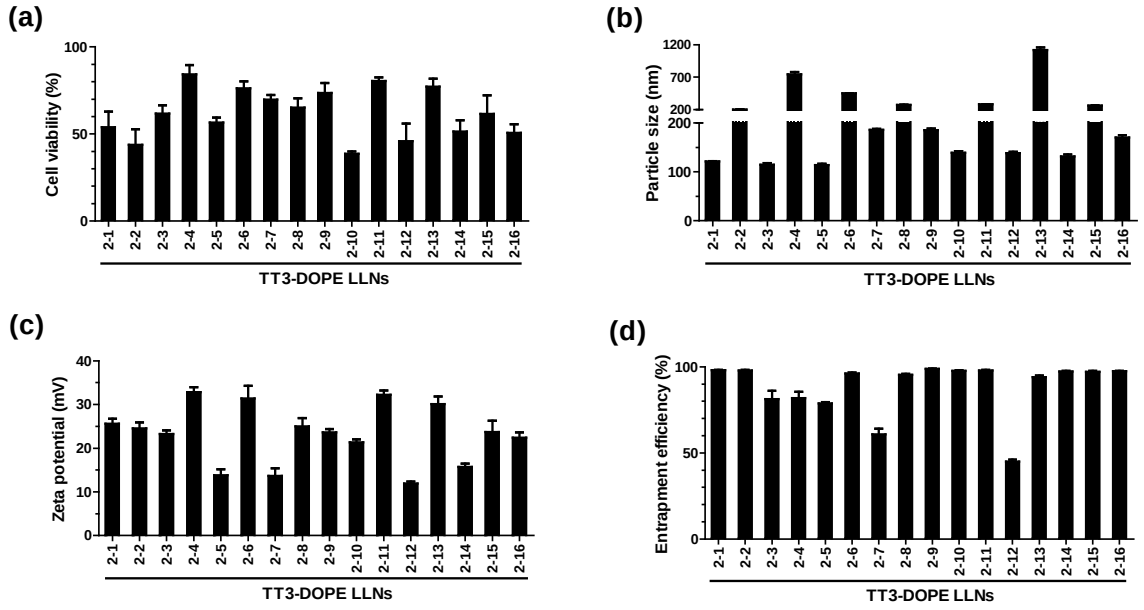

Figure S5. The cell viability (a), particle size (b), zeta potential (c), and entrapment efficiency (d) of **TT3**-DOPE LLNs formulated through the second round of orthogonal array.

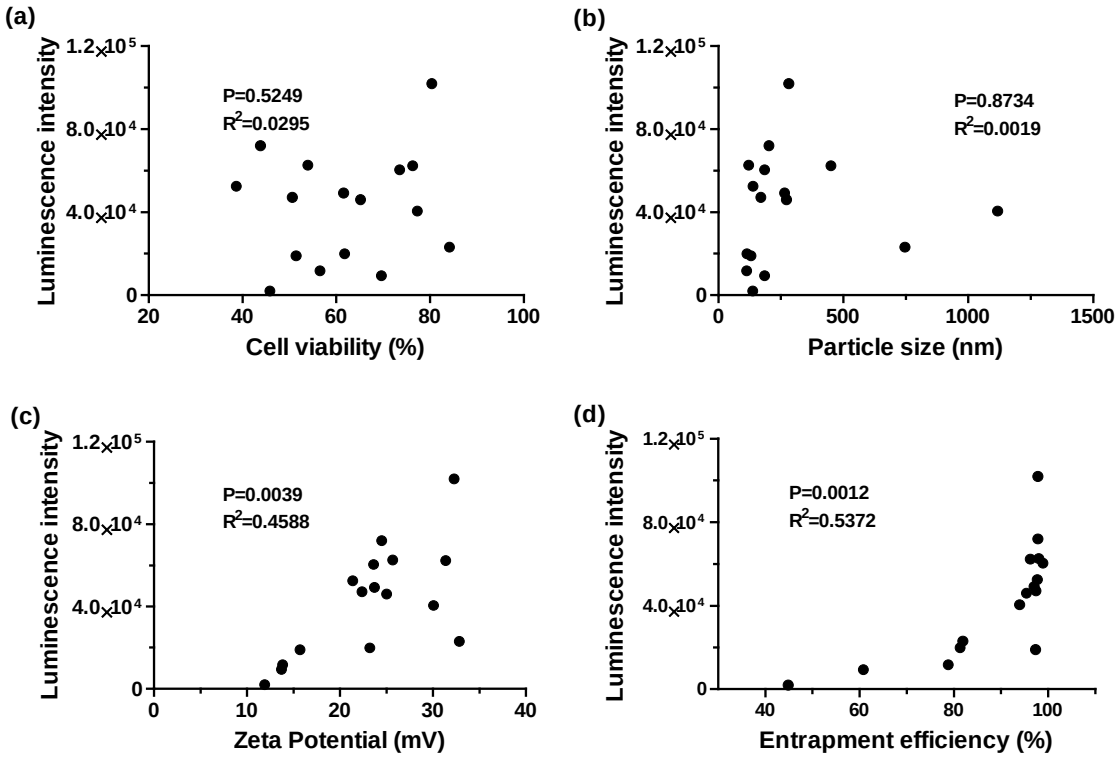

Figure S6. The correlation between transfection efficiency of **TT3**-DOPE LLNs formulated through the second round of orthogonal array and their corresponding cell viability, particle size, zeta potential, and entrapment efficiency.

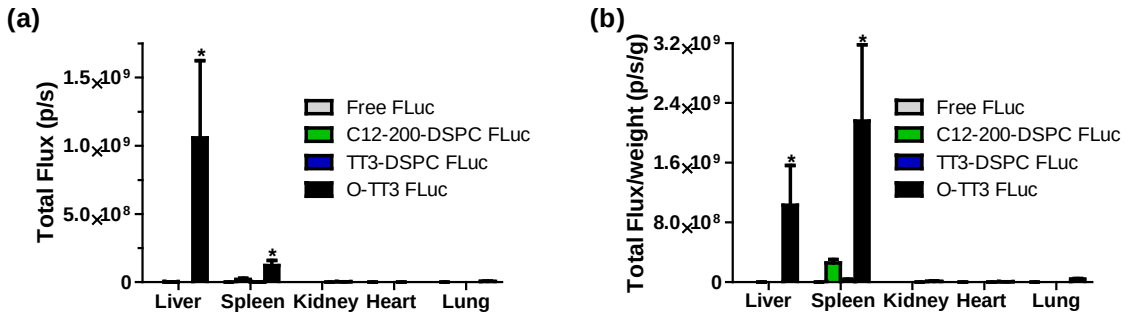

Figure S7. Bioluminescence signal (6 hours after administration in C57BL/6 mice). Free FLuc mRNA served as a negative control. Total bioluminescence signal (a) and normalized bioluminescence signal with tissue weight (b). Statistically significant difference was observed in the groups (*: $P < 0.05$; O-**TT3** FLuc vs C12-200-DSPC Fluc; **O-TT3** FLuc vs **TT3**-DSPC FLuc).

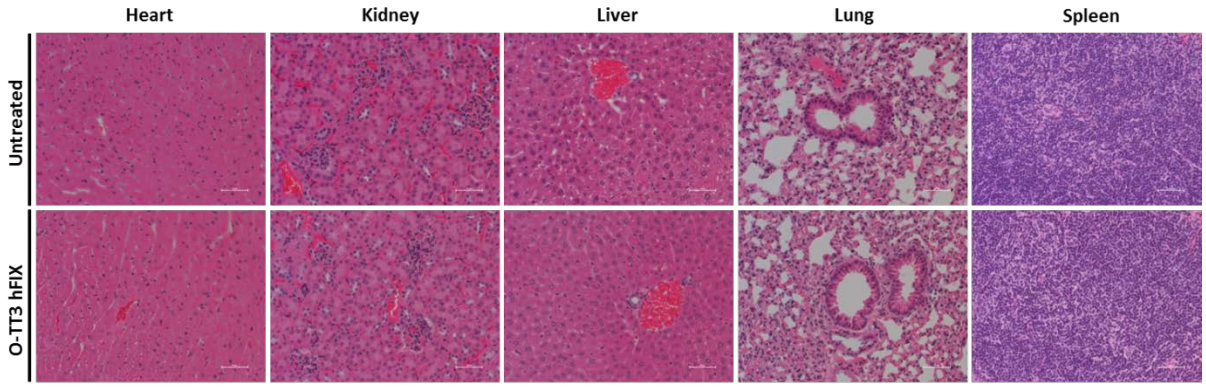

Figure S8. Histological analysis of major organs (heart, kidney, liver, lung, and spleen) after treatment of **O-TT3** hFIX. Untreated groups served as negative controls. No significant alteration of histology was observed in the **O-TT3** LLNs treated groups compared to the control groups.

Table S1. Orthogonal array table $L_{16}(4^4)$ (a) and K_n values (b) for the first round of **TT3**-DOPE LLNs.

(a) Orthogonal array table $L_{16}(4^4)$

TT3-DOPE LLNs	Formulation components (mole ratio)				Relative luminescence intensity
	TT3	DOPE	Cholesterol	DMG-PEG$_{2000}$	
1-1	30	2.5	38.5	3	1216
1-2	40	10	18.5	1.5	351
1-3	50	10	38.5	6	418
1-4	60	2.5	18.5	0.75	20
1-5	30	5	18.5	6	28
1-6	40	1.25	38.5	0.75	28
1-7	50	1.25	18.5	3	34
1-8	60	5	38.5	1.5	51
1-9	30	1.25	48.5	1.5	1207
1-10	40	5	28.5	3	203
1-11	50	5	48.5	0.75	469
1-12	60	1.25	28.5	6	30
1-13	30	10	28.5	0.75	4663
1-14	40	2.5	48.5	6	220
1-15	50	2.5	28.5	1.5	20
1-16	60	10	48.5	3	243

(b) K_n values

	TT3	DOPE	Cholesterol	DMG-PEG$_{2000}$
K_1^*	**1779**	325	108	**1295**
K_2^*	201	369	**1229**	407
K_3^*	235	188	428	424
K_4^*	86	**1419**	535	174
ΔK^{**}	1693	1231	1121	1121

$K_n^* = \Sigma RLI_n/4$

$\Delta K^{**} = K_{max} - K_{min}$

The impact of the four components on mRNA delivery (ΔK): **TT3** > DOPE > Cholesterol = DMG-PEG$_{2000}$.

Table S2. Orthogonal array table $L_{16}(4^4)$ (a) and K_n values (b) for the second round of **TT3**-DOPE LLNs.

(a) Orthogonal array table $L_{16}(4^4)$

TT3-DOPE LLNs	Formulation components (mole ratio)				Relative luminescence intensity
	TT3	DOPE	Cholesterol	DMG-PEG_{2000}	
2-1	10	20	35	0.15	62542
2-2	20	40	25	0.03	71935
2-3	30	40	35	0.75	19869
2-4	40	20	25	0	23096
2-5	10	30	25	0.75	11687
2-6	20	10	35	0	62216
2-7	30	10	25	0.15	9394
2-8	40	30	35	0.03	45962
2-9	10	10	40	0.03	60333
2-10	20	30	30	0.15	52468
2-11	30	30	40	0	101863
2-12	40	10	30	0.75	1917
2-13	10	40	30	0	40410
2-14	20	20	40	0.75	18962
2-15	30	20	30	0.03	49102
2-16	40	40	40	0.15	47049

(b) K_n values

	TT3	DOPE	Cholesterol	DMG-PEG_{2000}
K_1^*	43743	33465	29028	**56896**
K_2^*	**51395**	38426	35974	56833
K_3^*	45057	**52995**	47647	42863
K_4^*	29506	44816	**57052**	13109
ΔK^{**}	21889	19529	18025	43788

$K_n^* = \Sigma RLI_n/4$

$\Delta K^{**} = K_{max} - K_{min}$

The most efficient formulation in the second round of optimization was validated with a designated code of **Hi-TT3** LLNs (formulation ratio is **TT3**/DOPE/Chol/DMG-PEG_{2000}=20/30/40/0).

References

1. Matsuura, K.; Murasato, K.; Kimizuka, N. *J. Am. Chem. Soc.* **2005**, *127*, 10148-10149.