Supporting Information

Deciphering the Role of PEGylation on the Lipid Nanoparticle-Mediated mRNA Delivery to the Liver

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Table S1. Polydispersity index (PDI) of LNP library. Data is presented as mean ± SD (n = 3).

Items	PEG-lipid ratio (%)	PDI			Average	SD
DSPE LNP	1.5	0.207	0.188	0.183	0.193	0.013
	2.5	0.231	0.295	0.256	0.260	0.032
	5.0	0.233	0.262	0.261	0.250	0.016
DMG LNP	1.5	0.259	0.193	0.264	0.240	0.040
	2.5	0.233	0.210	0.268	0.240	0.029
	5.0	0.290	0.224	0.303	0.270	0.042
SA LNP	1.5	0.272	0.263	0.261	0.270	0.006
	2.5	0.193	0.245	0.276	0.240	0.042
	5.0	0.210	0.296	0.266	0.260	0.044

Table S2. Zeta potential of LNPs in diluted PBS (1×). Data is presented as mean ± SD (n = 3).

Items	PEG-lipid ratio (%)	Zeta (mV)			Average	SD
DSPE LNP	1.5	-8.17	-9.83	-9.34	-9.11	0.85
	2.5	-10.22	-9.42	-9.60	-9.75	0.42
	5.0	-5.49	-8.60	-7.64	-7.24	1.59
DMG LNP	1.5	-4.96	-5.91	-8.90	-6.59	2.06
	2.5	5.71	-2.51	1.43	1.54	4.11
	5.0	-7.41	4.95	-4.61	-2.36	6.48
SA LNP	1.5	-2.59	-6.13	-6.40	-5.04	2.13
	2.5	-3.28	-3.71	-0.93	-2.64	1.50
	5.0	-2.28	-2.61	-5.68	-3.52	1.88

Table S3. Encapsulation efficiency (EE) of mRNA by different LNPs. Data are presented as mean ± SD (n = 3).

Items	PEG-lipid ratio (%)	EE (%)			Average	SD
DSPE LNP	1.5	89.93	90.91	90.7	90.51	0.51
	2.5	89.9	90.19	89.84	89.98	0.19
	5.0	87.38	87.91	88.83	88.04	0.73
DMG LNP	1.5	87.39	89.85	90.08	89.11	1.49
	2.5	88.55	91.41	89.34	89.76	1.48
	5.0	92.3	92.57	93.08	92.65	0.4
SA LNP	1.5	90.81	91.51	92.17	91.5	0.68
	2.5	87.75	89.31	87.32	88.13	1.05
	5.0	86.99	81.11	88.53	85.54	3.91

Table S4. Characterization of DSPE LNP, DMG LNP and SA LNP loading Fluc mRNA and N.C. siRNA, respectively. PEG-lipid ratio for all LNPs is 1.5% and N:P ratio is 6. Data are presented as mean ± SD (n = 3).

LNP	Loading	Hydrodynamic diameter (nm)	PDI	Zeta (mV)	Encapsulation efficiency (%)
DSPE LNP	mRNA	125.60 ± 4.19	0.221 ± 0.028	-7.71 ± 1.88	91.49 ± 0.32
	siRNA	118.71 ± 11.32	0.179 ± 0.043	-2.48 ± 4.02	87.97 ± 0.93
DMG LNP	mRNA	112.07 ± 16.57	0.223 ± 0.082	-9.20 ± 1.89	92.18 ± 0.31
	siRNA	105.82 ± 10.88	0.263 ± 0.043	-1.66 ± 1.00	90.86 ± 0.21
SA LNP	mRNA	133.68 ± 11.63	0.232 ± 0.053	-8.89 ± 0.99	89.82 ± 0.61
	siRNA	115.77 ± 5.03	0.268 ± 0.029	-3.41 ± 1.80	88.80 ± 0.15

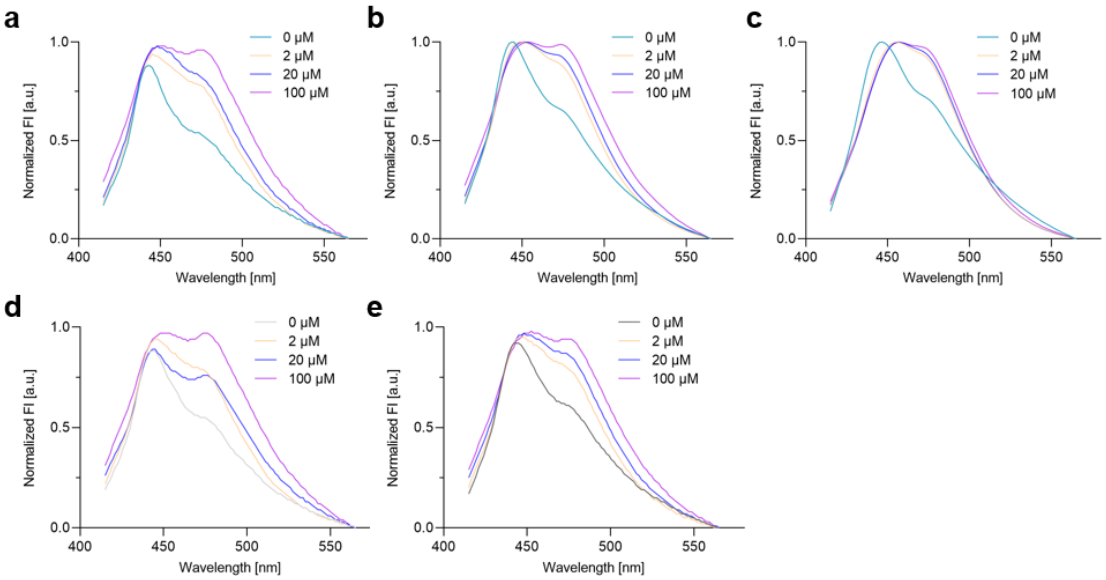

Figure S1. Assessment of human serum albumin (HSA) effect on LNP with Laurdan as the probe. The emission spectra of Laurdan-labeled LNPs upon incubation with different concentration (0 µM, 2 µM, 20 µM and 100 µM) of human serum albumin (HSA). **a)** DSPE LNP (1.5% DSPE-PEG); **b)** DSPE LNP (2.5% DSPE-PEG); **c)** DSPE LNP (5.0% DSPE-PEG); **d)** DMG LNP (1.5% DMG-PEG); **e)** SA LNP (1.5% SA-PEG).

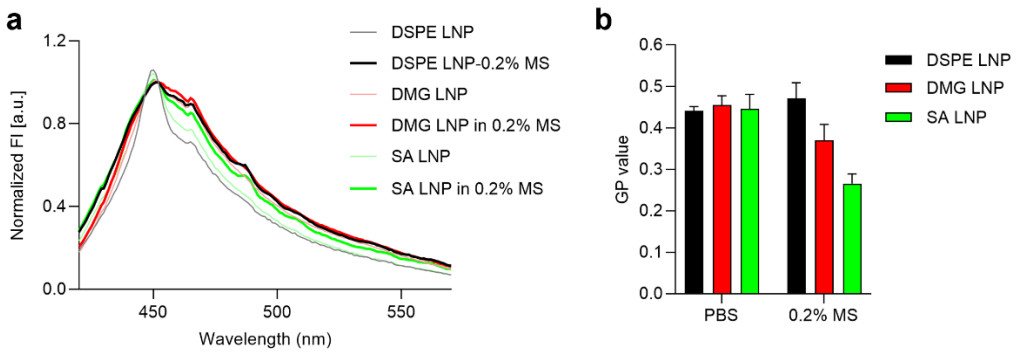

Figure S2. Assessment of mouse serum effect on LNP with Laurdan as the probe. **a)** The emission spectra of Laurdan-labeled LNPs (DSPE LNP, DMG LNP, and SA LNP containing 1.5% molar ratio of PEG-lipid) upon incubation with PBS and 0.2% mouse serum (MS). **b)** The influence of PBS and 0.2% Mouse serum (MS) on the GP values of DSPE LNP, DMG LNP, and SA LNP with a constant 1.5% PEG for all LNPs. Data are presented as mean ± SD (n = 3).

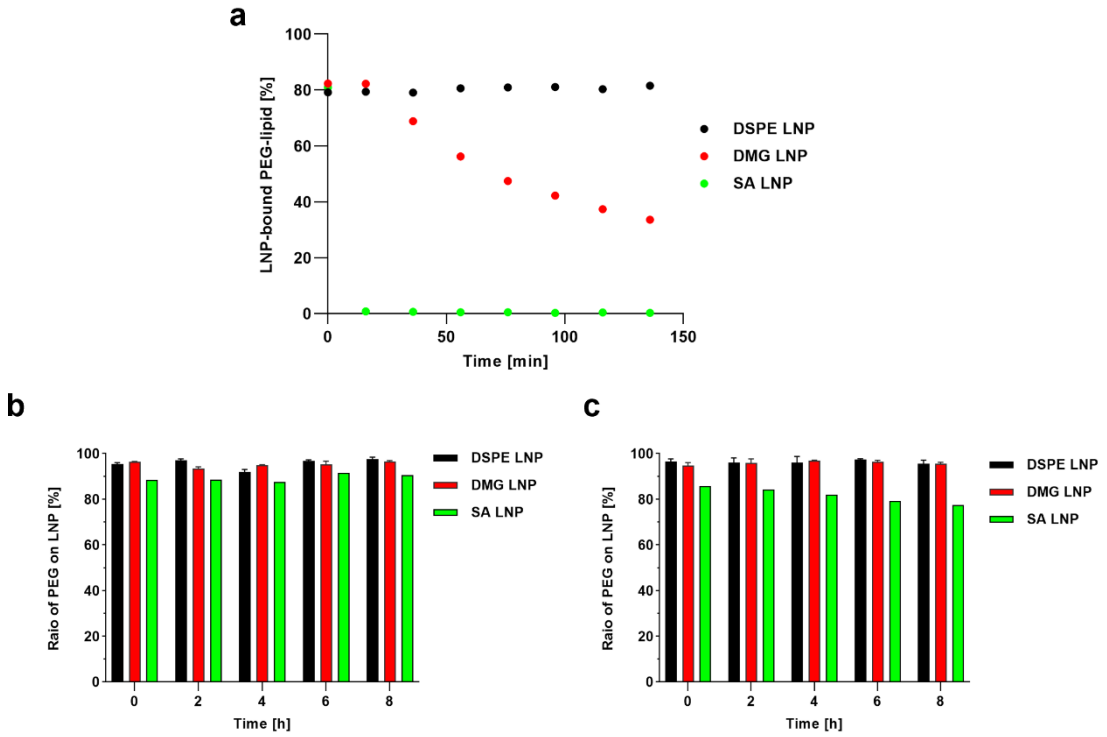

Figure S3. PEG dissociation from three LNPs. a) The remaining PEG on the surface of three LNPs (DSEP LNP, DMG LNP and SA LNP containing 1.5% PEG lipids) incubated with 40% mouse serum (MS) and monitored within 2 h for PGSE-NMR measurement. PEG shedding kinetics of three LNPs incubated with **b)** serum-free media (PBS solution) and **c)** 10% MS were monitored within 9 h by PGSE-NMR measurement. Data are presented as mean ± SD (n = 3).

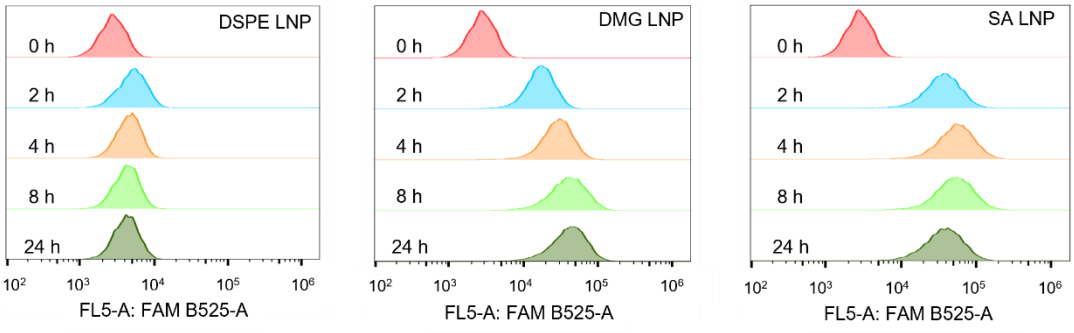

Figure S4. Flow cytometry analysis of kinetic LNP uptake in Huh-7 cells. DSPE LNP (left), DMG LNP (middle), and SA LNP (right). The background fluorescence intensity for untreated cells is 2.9×10^3.

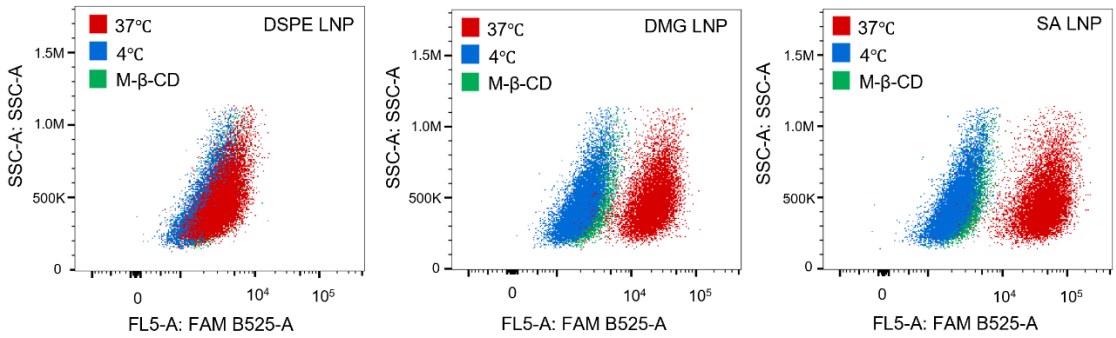

Figure S5. The influence of incubation temperature and methyl beta cyclodextrin (M-β-CD) on the LNP internalization in Huh-7 cells after 2 h's incubation. The background fluorescence intensity for untreated cells is 2.1×10^3.

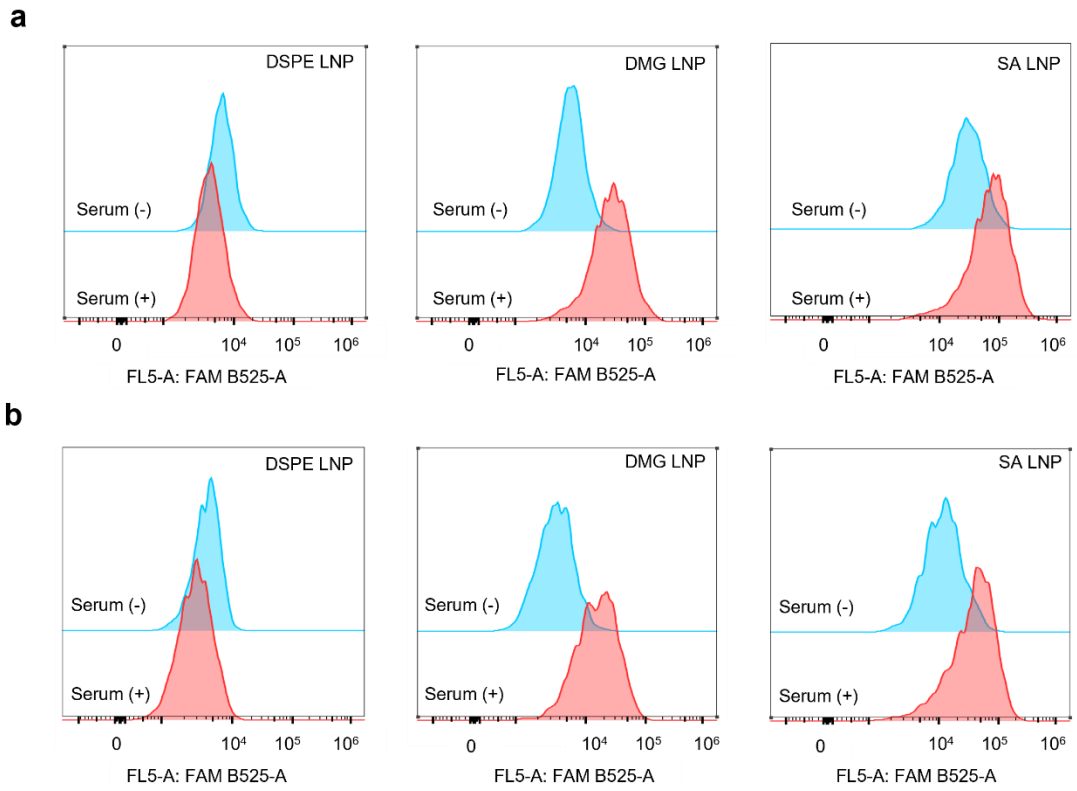

Figure S6. Flow cytometry analysis of LNP uptake in a) Huh-7 cells and b) HepG2 cells 4 h's incubation in serum-containing (red) and serum-free (cyan) medium. The background fluorescence intensity of untreated HepG2 cells is 3.1×10^3 and untreated Huh-7 cells is 5.0×10^3.

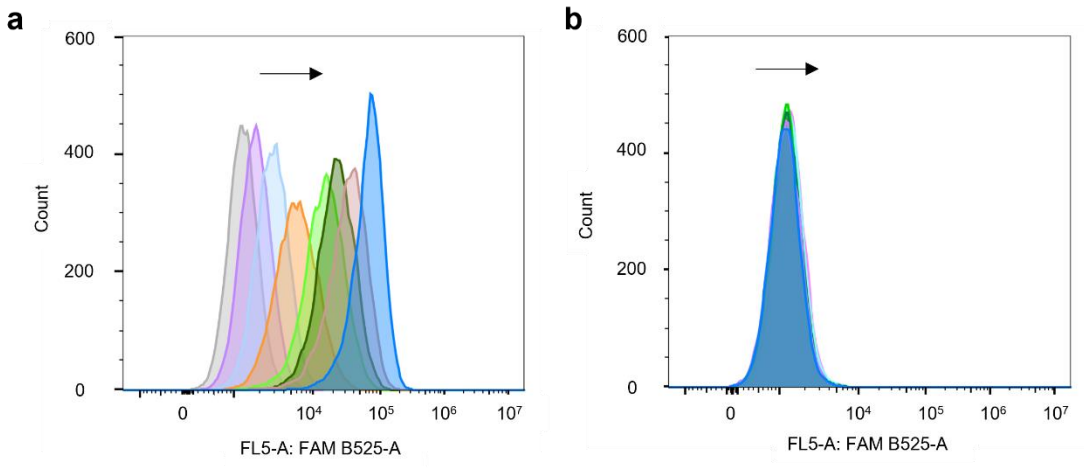

Figure S7. Transient stabilization by PEGylated lipid is essential for LNP delivery. FAM-labeled siRNA-loaded **a)** SA LNP and **b)** PEG-free LNP showed different particle uptake by Huh-7 cells upon incubation for 0 min, 2 min, 5 min, 15 min, 30 min, 45 min, 60 min and 120 min, respectively (*left* to *right*).

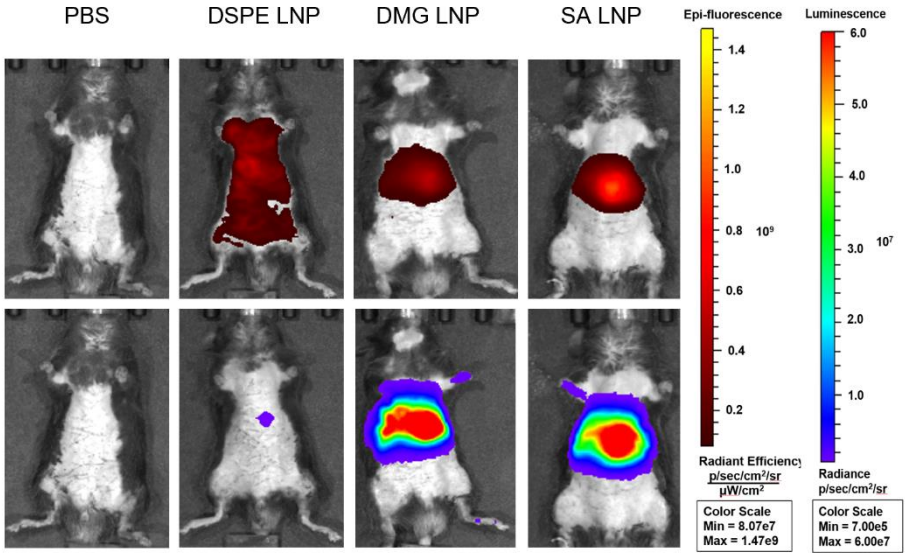

Figure S8. Representative images of DiR fluorescence (*top*) and Fluc bioluminescence (*bottom*) at 4 h post intravenous injection of different formulations, including PBS, DSPE LNP, DMG LNP, and SA LNP. All nanoparticles were labeled with DiR and Fluc mRNA was encapsulated therein.

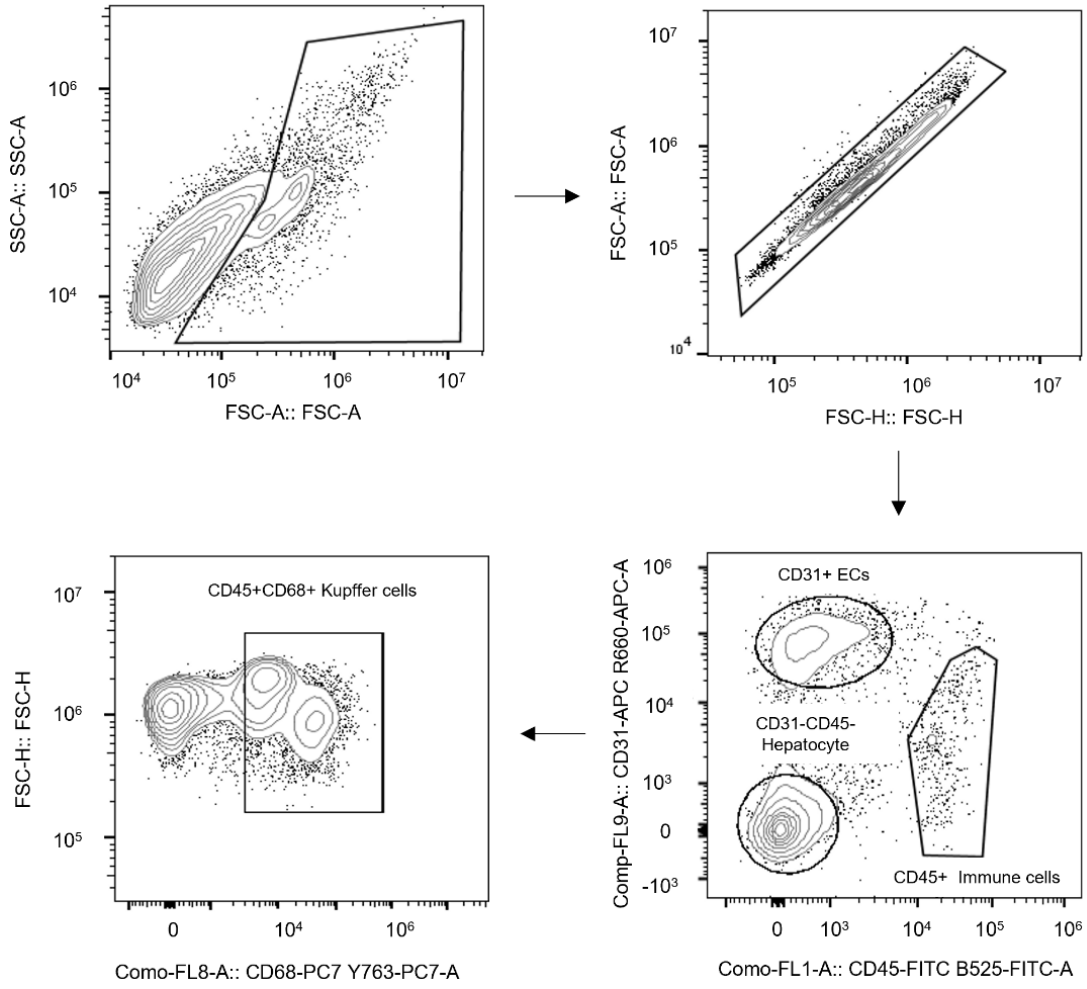

Figure S9. Gating strategies for flow cytometry analysis of different types of liver cells.

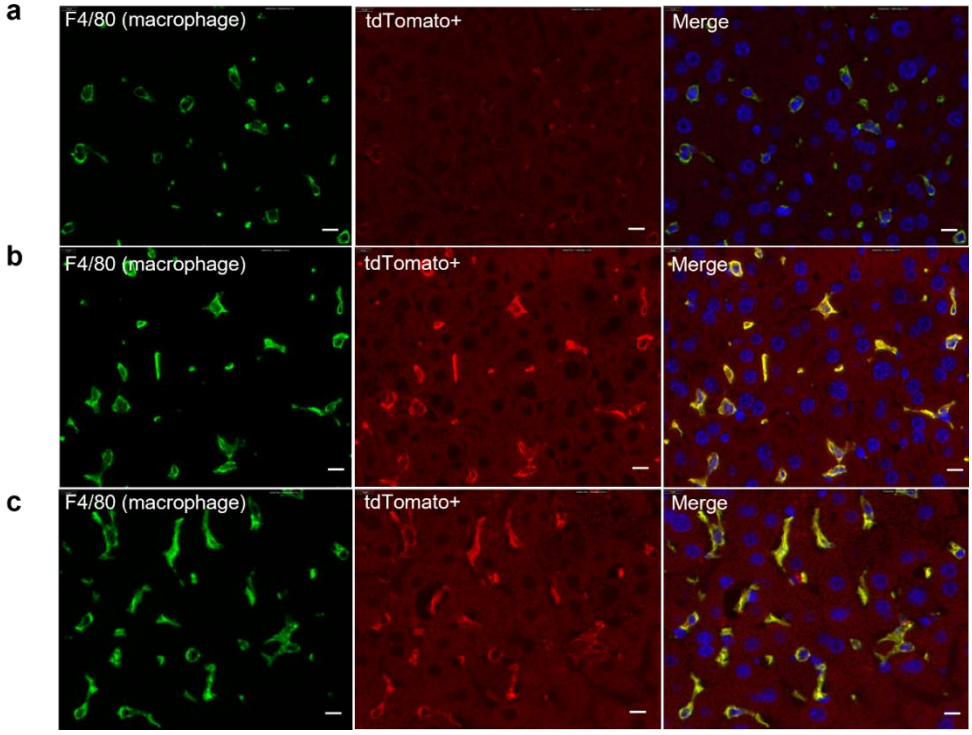

Figure S10. Immunofluorescence images of tdTomato$^+$ macrophages in liver tissue after treatment of three LNPs, **a) DSPE LNP, b) DMG LNP and c) SA LNP**. Green fluorescence refers to F4/80 antibody staining, and red fluorescence represents the tdTomato$^+$ signal, and blue fluorescence indicates the cell nucleus. Scale bar: 10 μm.

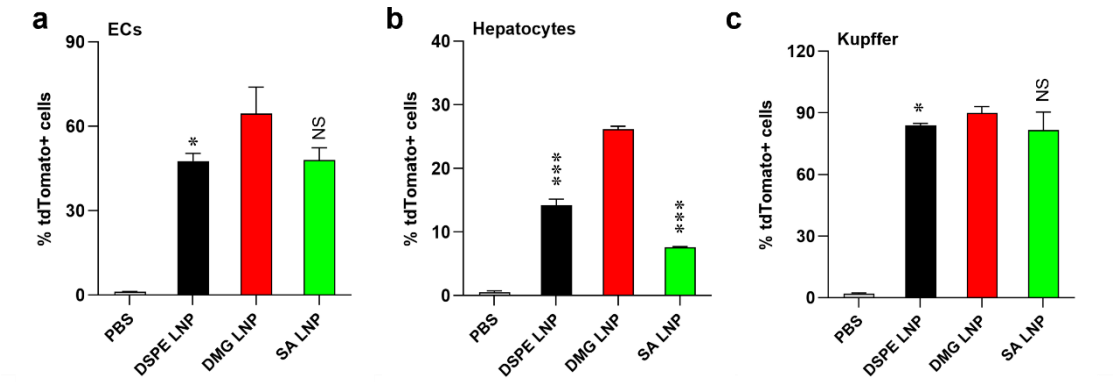

Figure S11. FACS analysis of ratios of tdTomato+ cells in ECs, hepatocytes and Kupffer cells 48 hours after intravenously injection of three LNPs. tdTomato+ ratios in **a)** ECs, **b)** hepatocytes and **c)** Kupffer cells were statistically concluded by specific markers identify and FACS analysis.

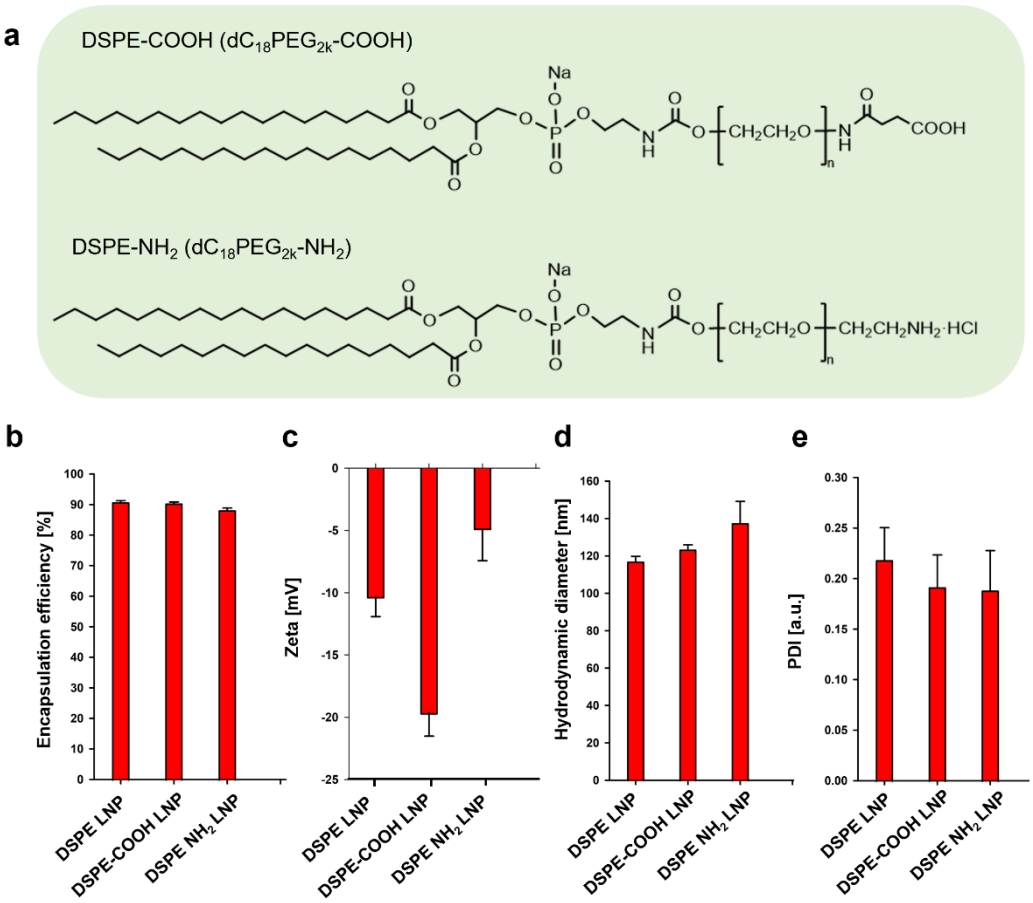

Figure S12. Physicochemical characterization of LNPs stabilized with different types of DSPE PEG (1.5%) terminated with methxyl, carboxyl and amine (n = 3). **a)** Chemical structure of carboxy- and amine-terminated DSPE PEG. The encapsulation efficiency of **b)** Fluc mRNA, **c)** zeta poential, **d)** hydrodynamic diameter, and **e)** polydispersity index (PDI) of three types of nanoparticles, DSPE LNP, DSPE-COOH LNP, and DSPE-NH_2 LNP.

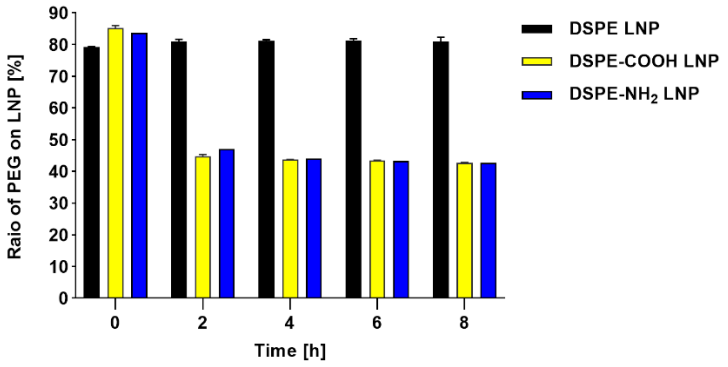

Figure S13. The remaining PEG on the surface of DSPE LNP, DSPE-COOH LNP and DSPE-NH_2 LNP (1.5% PEG) post 40% mouse serum incubation in 9 hours measured and analyzed with PGSE-NMR.

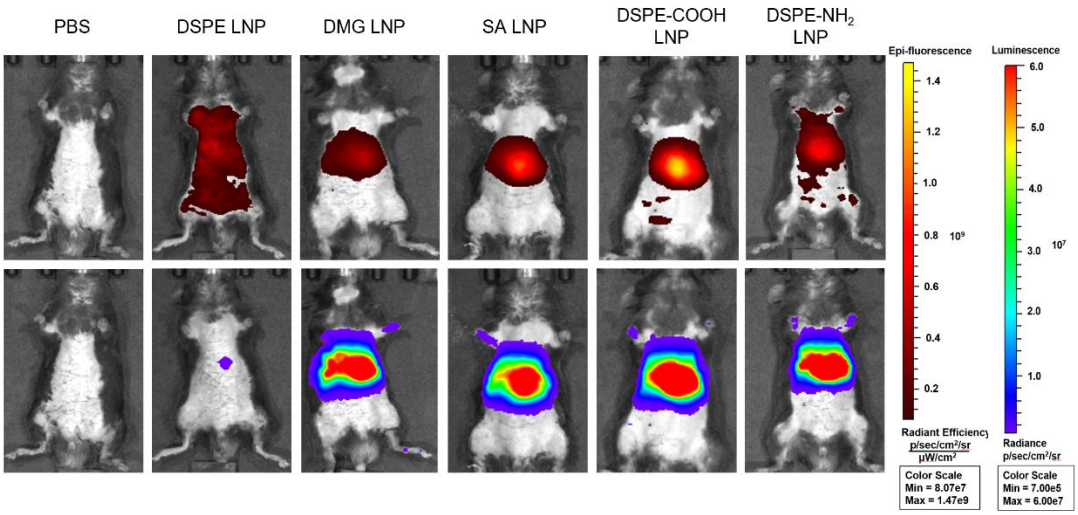

Figure S14. Comparison of liver targeting of five different lipid nanoparticles with PBS as the control (n = 3). Representative images of *in vivo* biodistribution of LNPs 4 h post administration, indexed by DiR fluorescence (*top*) and Fluc bioluminescence (*bottom*); Mice were intravenously injected with different groups (PBS, DSPE LNP, DSPE-COOH LNP, DSPE-NH_2 LNP, DMG LNP, and SA LNP. All LNPs were labeled with DiR and loaded with Fluc mRNA.

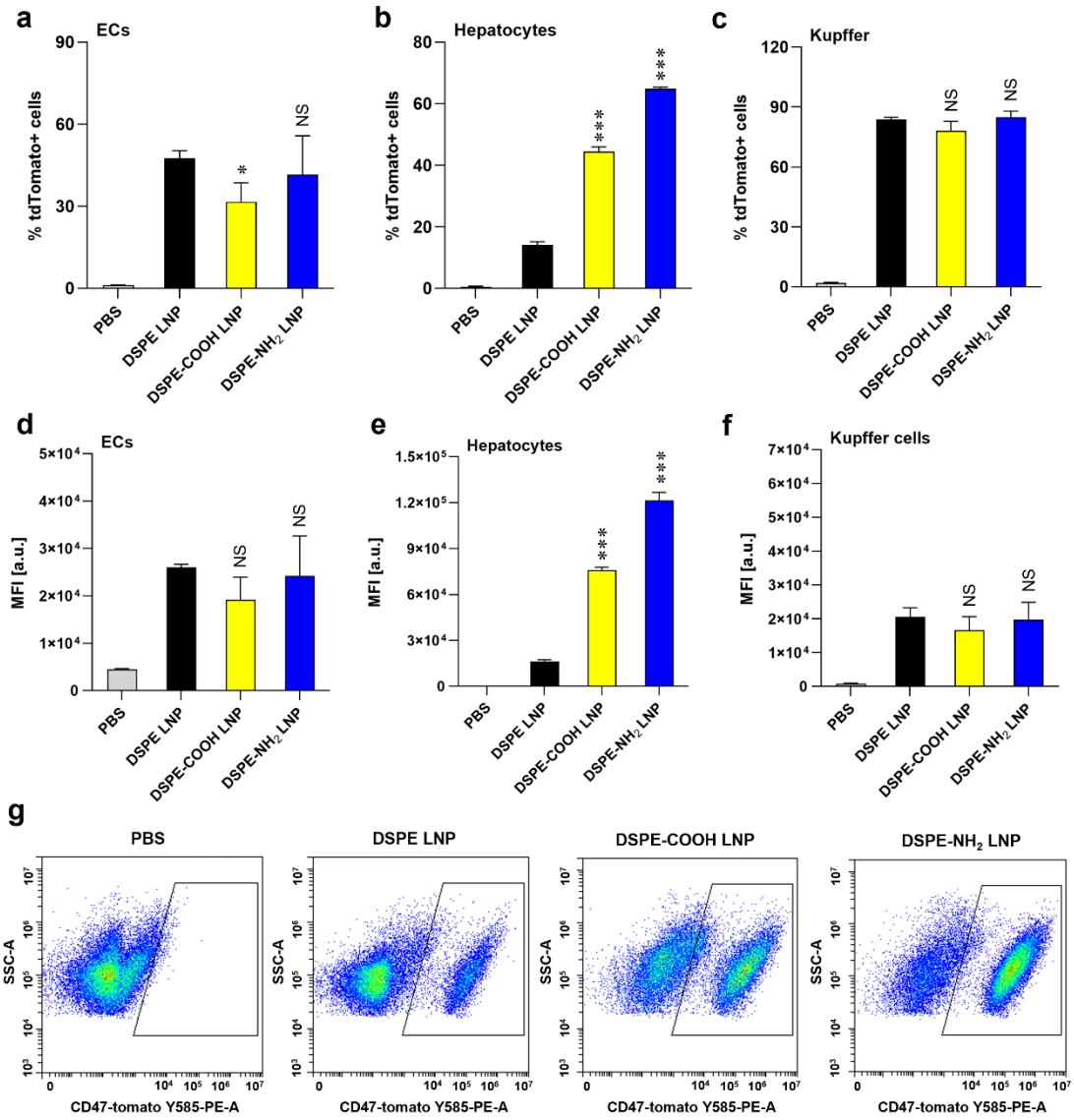

Figure S15. FACS analysis of tdTomato+ cells in ECs, hepatocytes and Kupffer cells after intravenous injection of DSPE LNP, DSPE-COOH LNP, and DSPE-NH_2 LNP. tdTomato+ ratios in **a)** ECs, **b)** hepatocytes and **c)** Kupffer cells were statistically concluded by specific markers identify and FACS analysis. The mean fluorescence intensity (MFI) in specific cells was shown in **d)** ECs, **e)** hepatocyte cells, and **f)** Kupffer cells, respectively. Statistical analysis was made with reference to DSPE LNP. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$. **g)** Representative FACS images of tdTomato+ cells in hepatocyte cellsin liver tissue of Ai14 mice 48 h post treatment by PBS, DSPE LNP, DSPE-COOH LNP, and DSPE-NH_2 LNP.