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Protocol

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mRNA lipid nanoparticle formulation, characterization and evaluation

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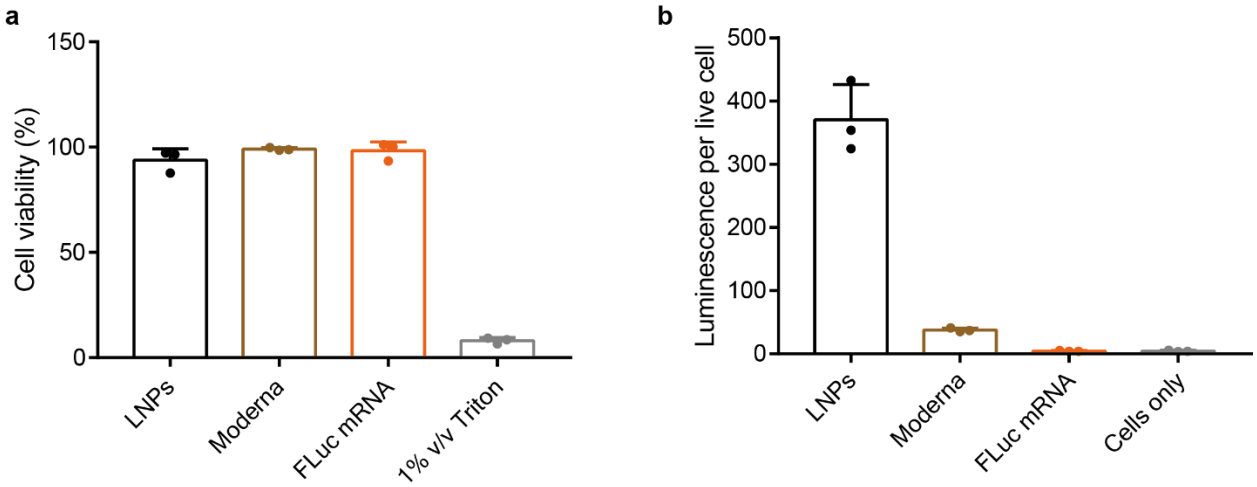
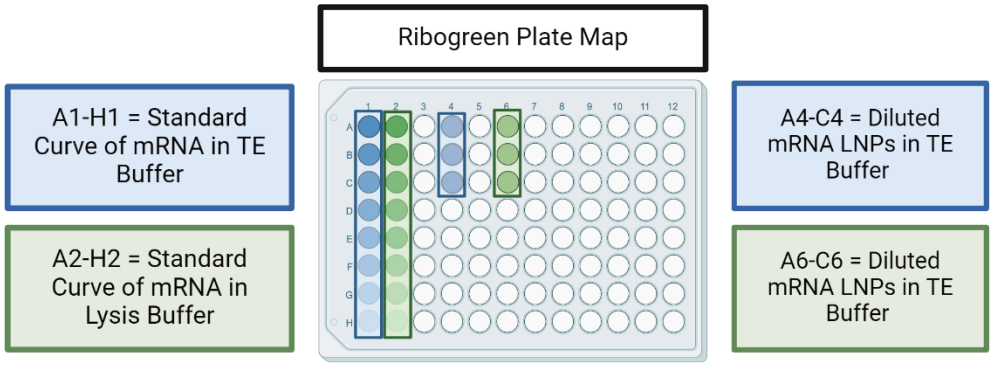


Supplementary Fig.1. *In vitro* evaluation methods of mRNA LNPs. (a), Cell viability and **(b),** *in vitro* FLuc expression of mRNA LNPs with Moderna control under 100 µL 500 ng/mL of total FLuc mRNA doses on HepG2 cells for 24 h (Data are shown for triplicates).



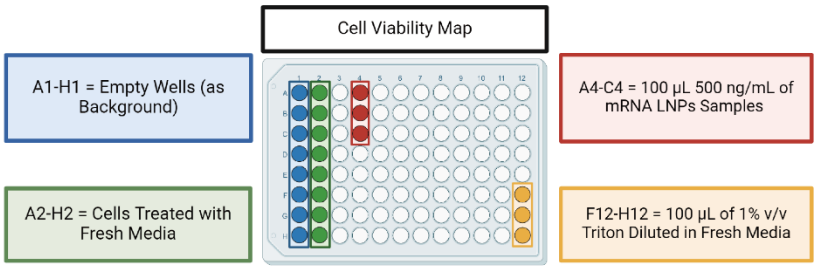
Standard RNA (µg/mL)	Standard Curve in TE Buffer (Relative Fluorescent Units)	Standard Curve in 2% v/v TE + Triton Buffer (Relative Fluorescent Units)	mRNA LNPs in TE Buffer (Relative Fluorescent Units)	mRNA LNPs in TE + 2% v/v Triton Buffer (Relative Fluorescent Units)
2	9896774	9931103	1083616	2549202
1	5277404	5334500	1175847	2550192
0.5	2919521	2948434	1207850	2533782
0.25	1659096	1838701		
0.125	1097696	1224094		
0.0625	774622	984824		
0.03125	627578	828294		
0	470298	645723		

Standard Curve of mRNA in TE Buffer		Standard Curve of mRNA in TE + 2% v/v Triton Buffer	
Y = 4719783 × X + 498919	R^2 = 0.9999	**Y = 4635939 × X + 667099**	R^2 = 0.9999

		mRNA LNPs in TE Buffer (µg/mL) Indicating Unencapsulated mRNA Concentration		mRNA LNPs in TE +2% v/v Triton Buffer (µg/mL) Indicating Total mRNA Concentration
Replicate 1	(1083616 - 498919) / 4719783 =	0.12388	(2549202 - 667099) / 4635939 =	0.40598
Replicate 2	(1175847 - 498919) / 4719783 =	0.14342	(2550192 - 667099) / 4635939 =	0.40619
Replicate 3	(1207850 - 498919) / 4719783 =	0.15020	(2533782 - 667099) / 4635939 =	0.40265

Encapsulation Efficiency (%)	65.6% ± 3.37%
Total mRNA Concentration	40.5 ± 0.002 µg/mL

Supplementary Fig.2. Example of step-by-step guide to quantifying mRNA encapsulation within mRNA LNPs (Data are shown for triplicates).



Wavelength 570 nm

Empty Wells (Relative Absorbance Units)	Cells Treated with Fresh Media (Relative Absorbance Units)	mRNA LNPs (Relative Absorbance Units)	1% v/v Triton in Fresh Media (Relative Absorbance Units)
0.583	0.676	0.693	0.594
0.572	0.631	0.69	0.522
0.576	0.659	0.676	0.522
0.566	0.65		
0.552	0.685		
0.568	0.691		
0.534	0.691		
0.51	0.673		

Wavelength 600 nm

Empty Wells (Relative Absorbance Units)	Cells Treated with Fresh Media (Relative Absorbance Units)	mRNA LNPs (Relative Absorbance Units)	1% v/v Triton in Fresh Media (Relative Absorbance Units)
0.531	0.384	0.393	0.536
0.516	0.393	0.412	0.472
0.521	0.385	0.381	0.472
0.532	0.354		
0.515	0.388		
0.524	0.39		
0.506	0.397		
0.501	0.382		

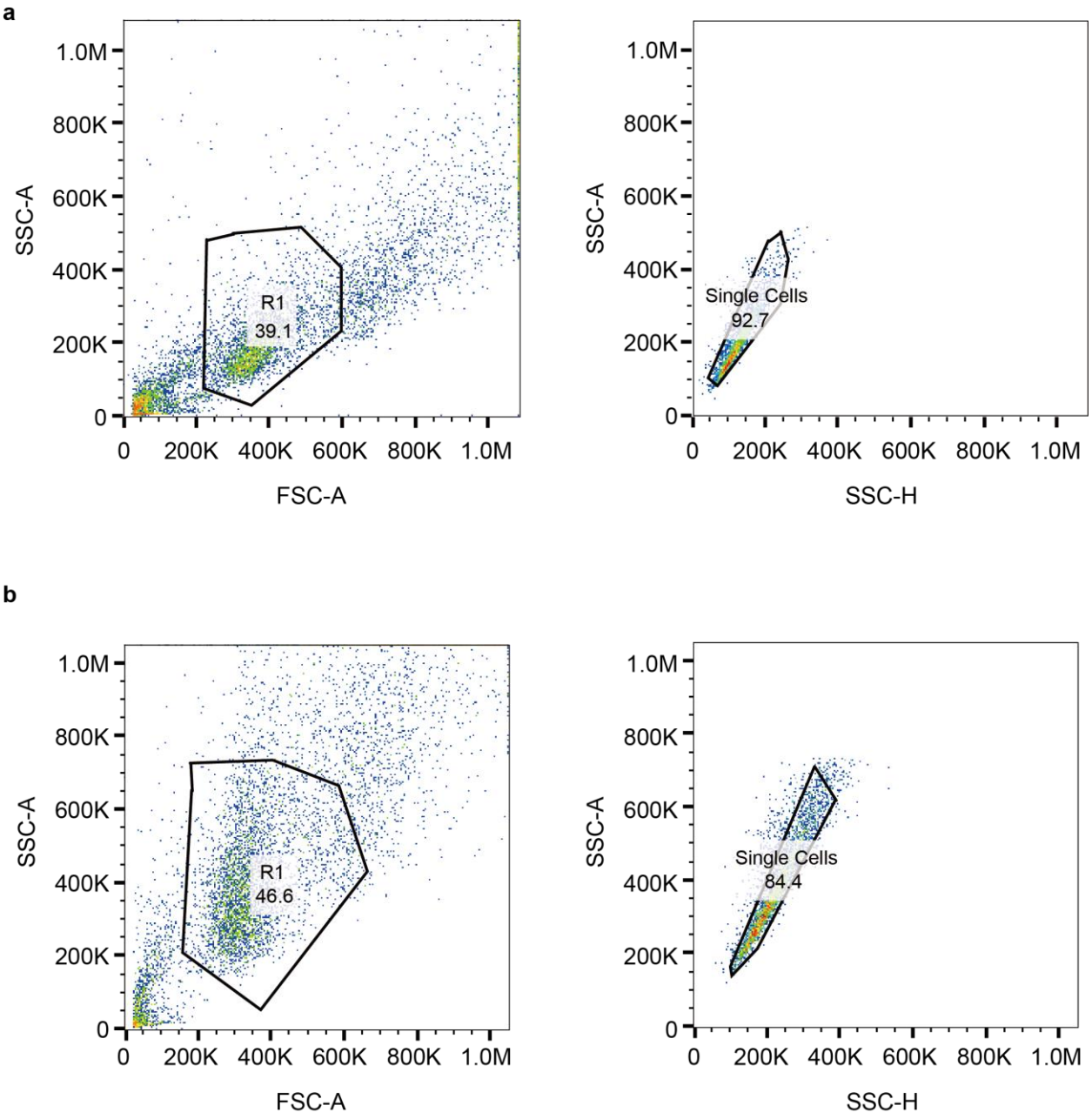
Wavelength 570 nm
-
Wavelength 600 nm

Empty Wells (Relative Absorbance Units)	Cells Treated with Fresh Media (Relative Absorbance Units)	mRNA LNPs (Relative Absorbance Units)	1% v/v Triton in Fresh Media (Relative Absorbance Units)
0.052	0.292	0.3	0.058
0.056	0.238	0.278	0.05
0.055	0.274	0.295	0.05
0.034	0.296		
0.037	0.297		
0.044	0.301		
0.028	0.294		
0.009	0.291		
Average Empty Wells	Average Cells treated with Fresh Media		
0.039375	0.285375		

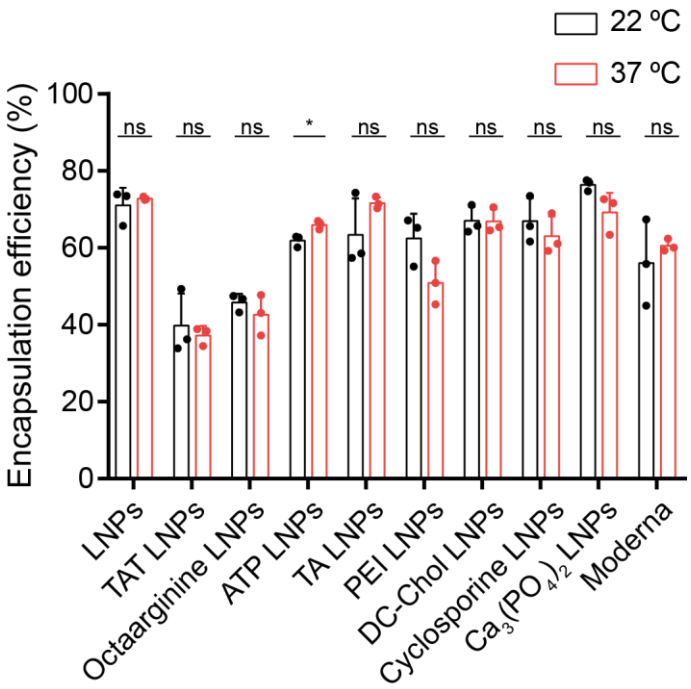
All Wells - Average Empty Wells

	Updated Average Cells Treated with Fresh Media	mRNA LNPs (Relative Absorbance Units)	1% v/v Triton in Fresh Media (Relative Absorbance Units)
	0.246	0.260625	0.018625
		0.238625	0.010625
		0.255625	0.010625
Cell viability (%)	mRNA LNPs		1% v/v Triton
0.260625 / 0.246 × 100% =	105.9451	**0.018625 / 0.246 × 100% =**	7.571138
0.238625 / 0.246 × 100% =	97.00203	**0.010625 / 0.246 × 100% =**	4.319106
0.255625 / 0.246 × 100% =	103.9126	**0.010625 / 0.246 × 100% =**	4.319106

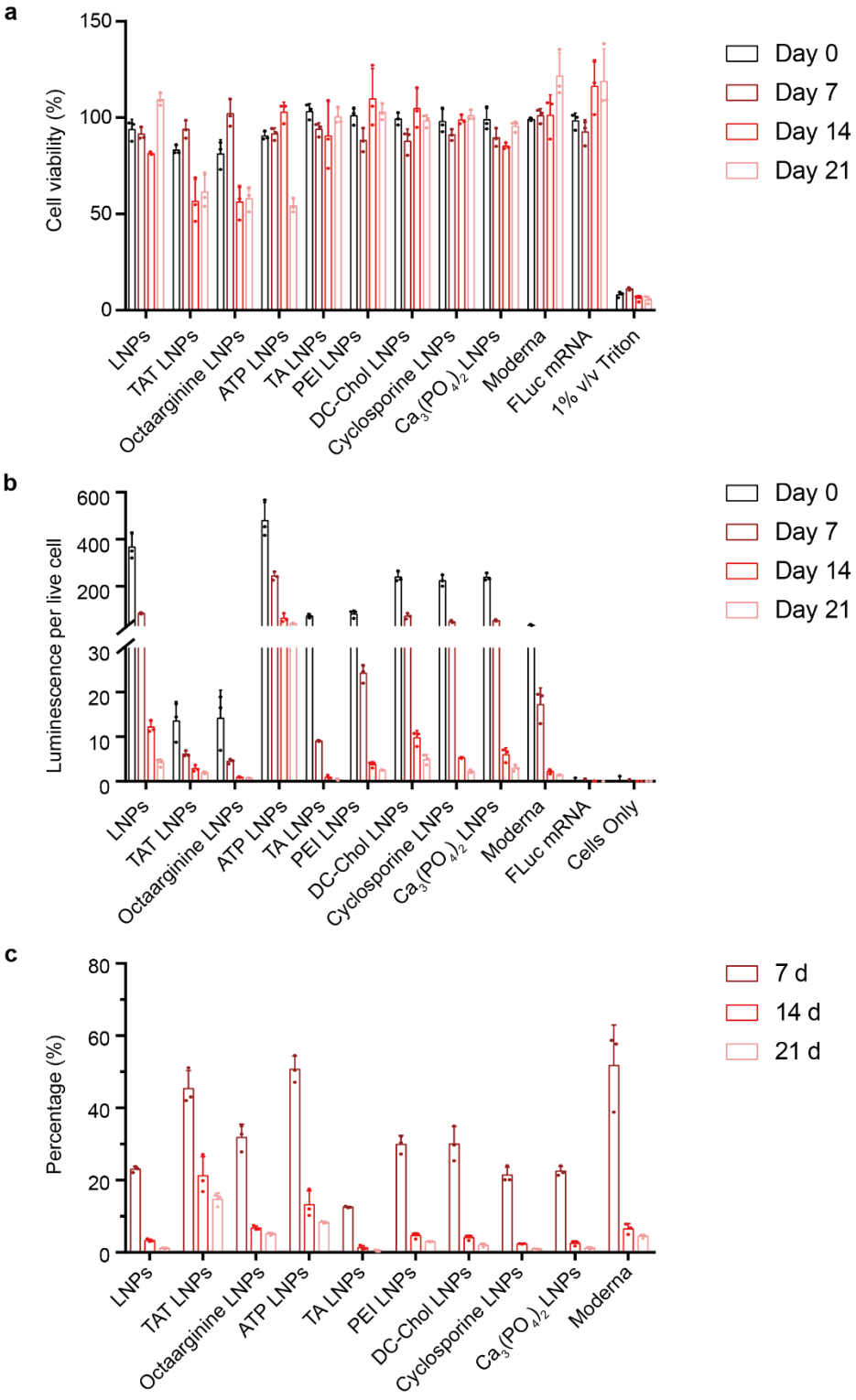
Supplementary Fig.3. Example of step-by-step guide to calculate cell viability using Alamar blue cell viability assay (Data are shown for triplicates).



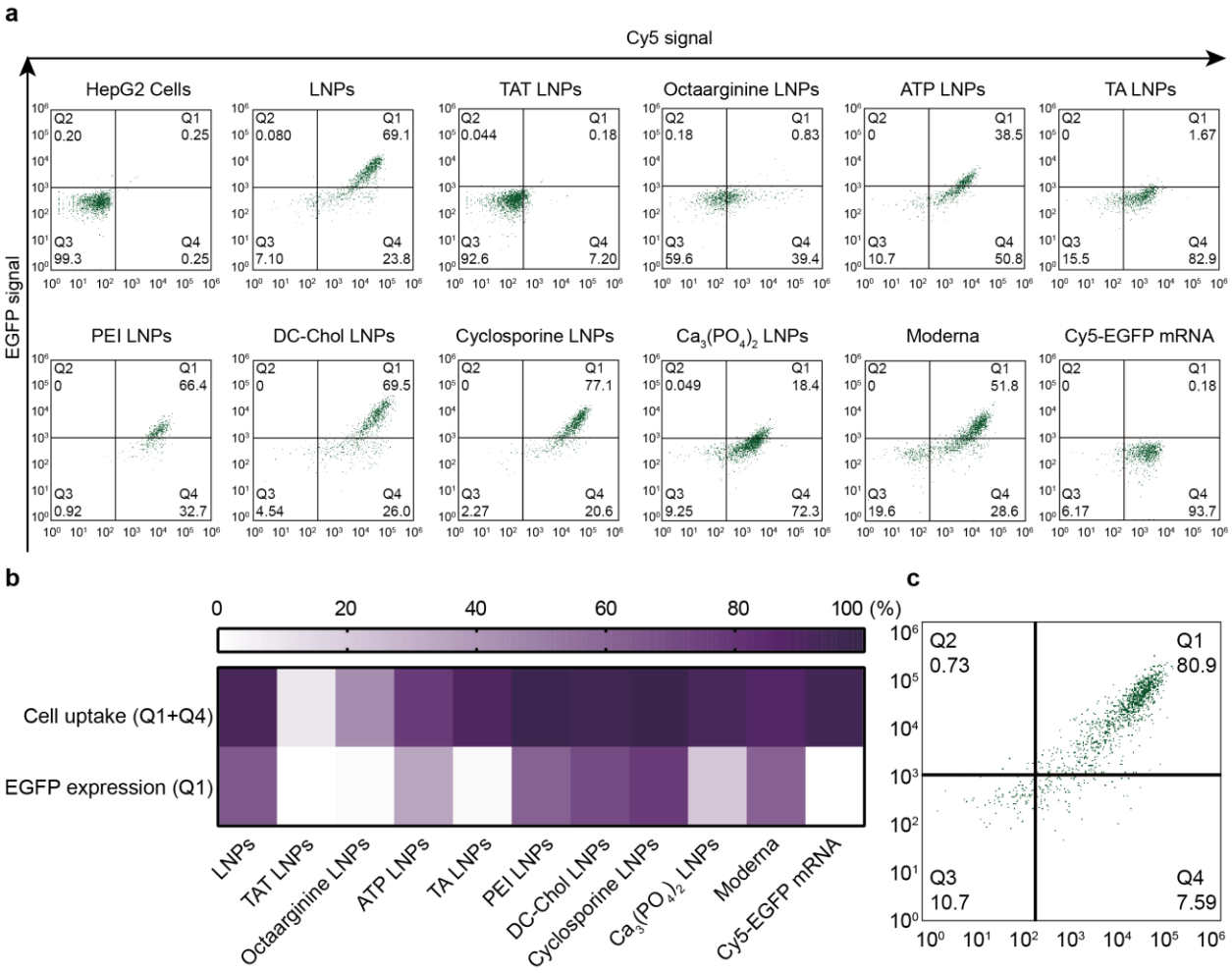
Supplementary Fig.4. Original gating to quantify the Cy5-EGFP expression at **(a),** 6 h and **(b),** 24 h for HePG2 cells



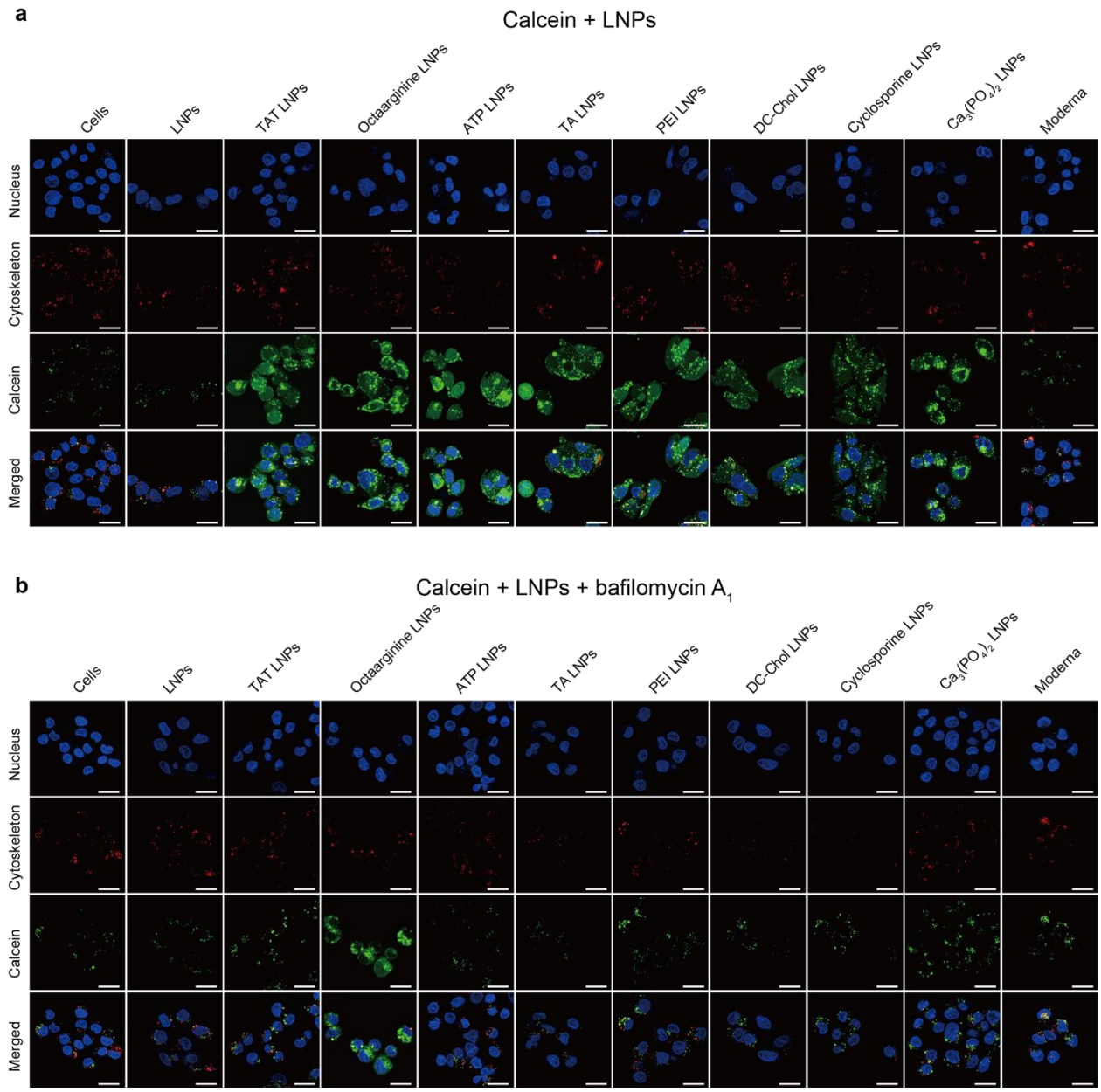
Supplementary Fig.5. mRNA encapsulation efficiency measurement at 22 °C and 37 °C *p < 0.05 and ns > 0.05 with 95% of confidence level from paired t-test (Data are shown for triplicates).



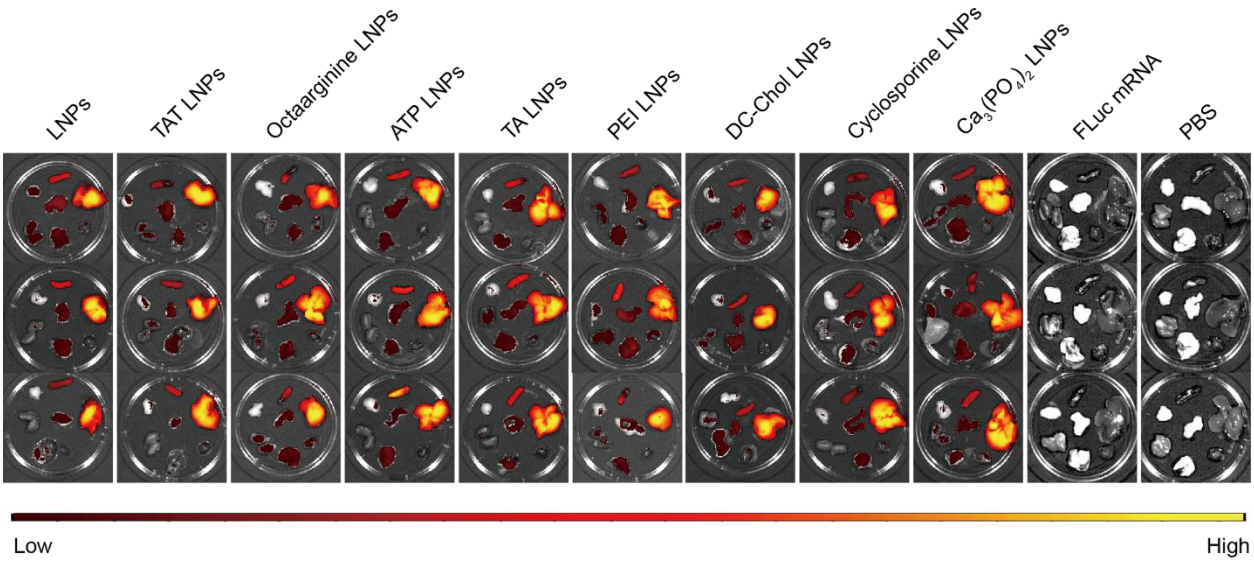
Supplementary Fig.6. (a), Cell viability and **(b),** normalized *in vitro* FLuc expression of mRNA LNPs with Moderna control under 100 µL 500 ng/mL of total FLuc mRNA doses on HepG2 cells over desired time (day 0, 7, 14 and 21). **(c),** The percentage of *in vitro* FLuc expression over desired time (7, 14 and 21) as compared to day 0 (Data are shown for triplicates).



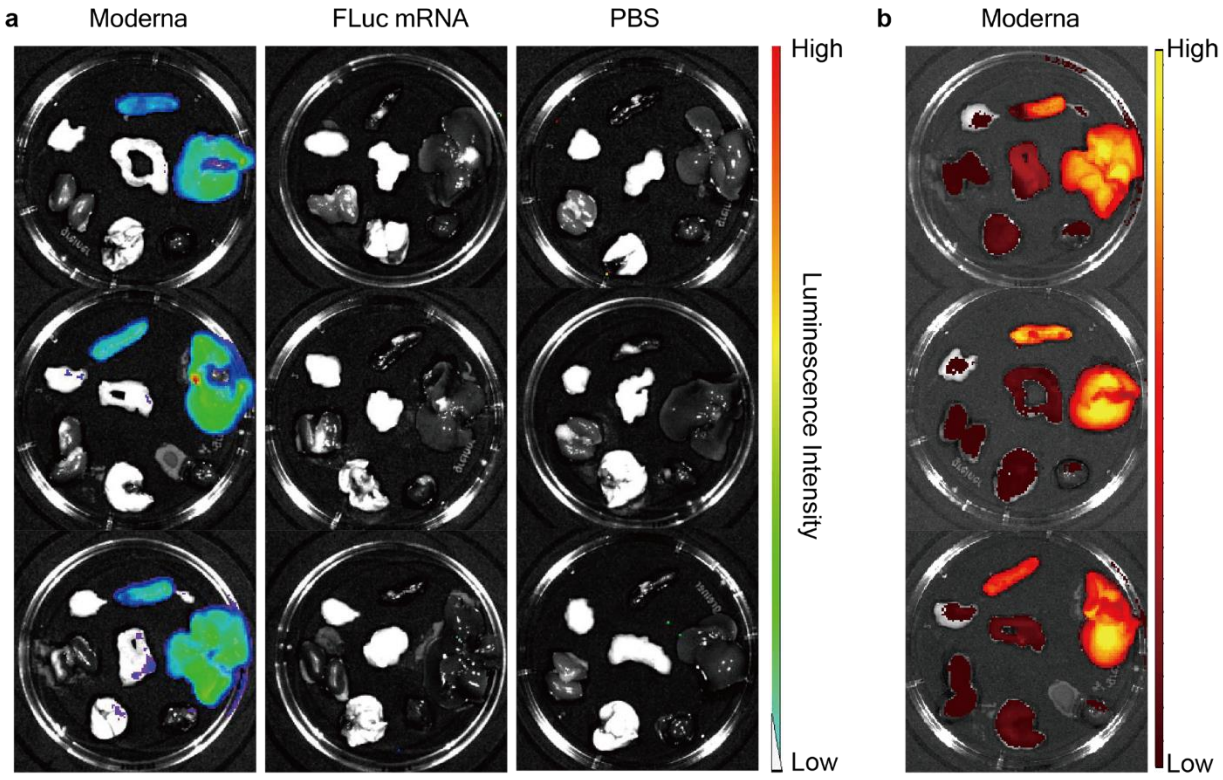
Supplementary Fig.7. (a), Representative data of EGFP signals vs. Cy5 signals on HepG2 cells incubated in the absence (control) or presence mRNA LNPs for 6 h at 37 °C. Q1 represents EGFP (+) and Cy5 (+) signal (mRNA LNPs uptake and EGFP expression), Q2 represents EGFP (+) and Cy5 (-) signal (false), Q3 represents EGFP (-) and Cy5 (-) signal (no mRNA LNPs and no EGFP expression), Q4 represents EGFP (-) and Cy5 (+) signal (mRNA LNPs uptake and no EGFP expression). **(b),** Heat-map summary of cell uptake and EGFP expression of HepG2 cells treated with Cy5-EGFP mRNA LNPs for 6 h at 37 °C. **(c),** Representative data of EGFP signals vs. Cy5 signals on HepG2 cells incubated in the absence (control) or presence mRNA LNPs with Moderna control for 24 h at 37 °C.



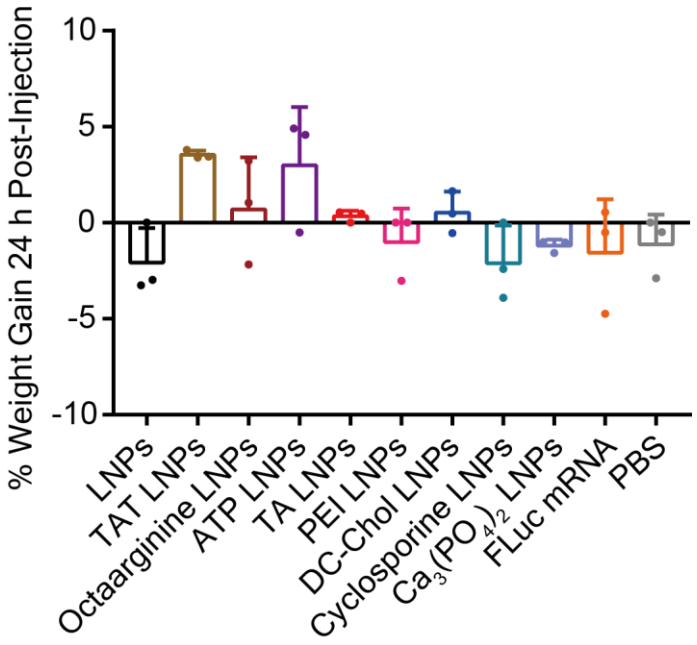
Supplementary Fig.8. Representative confocal images of HepG2 cells incubated with calcein or calcein and mRNA LNPs with nuclei and cytoskeletons staining in the **(a)** absence (top row) and **(b)** presence (bottom row) of inhibitor bafilomycin A_1 for 4 h at 37 °C are shown. Nuclei (blue) are stained with Hoechst 33342 and cytoskeletons are stained with CellMask™ Deep Red Actin Tracking Stain (red). Scale bars are 20 μm.



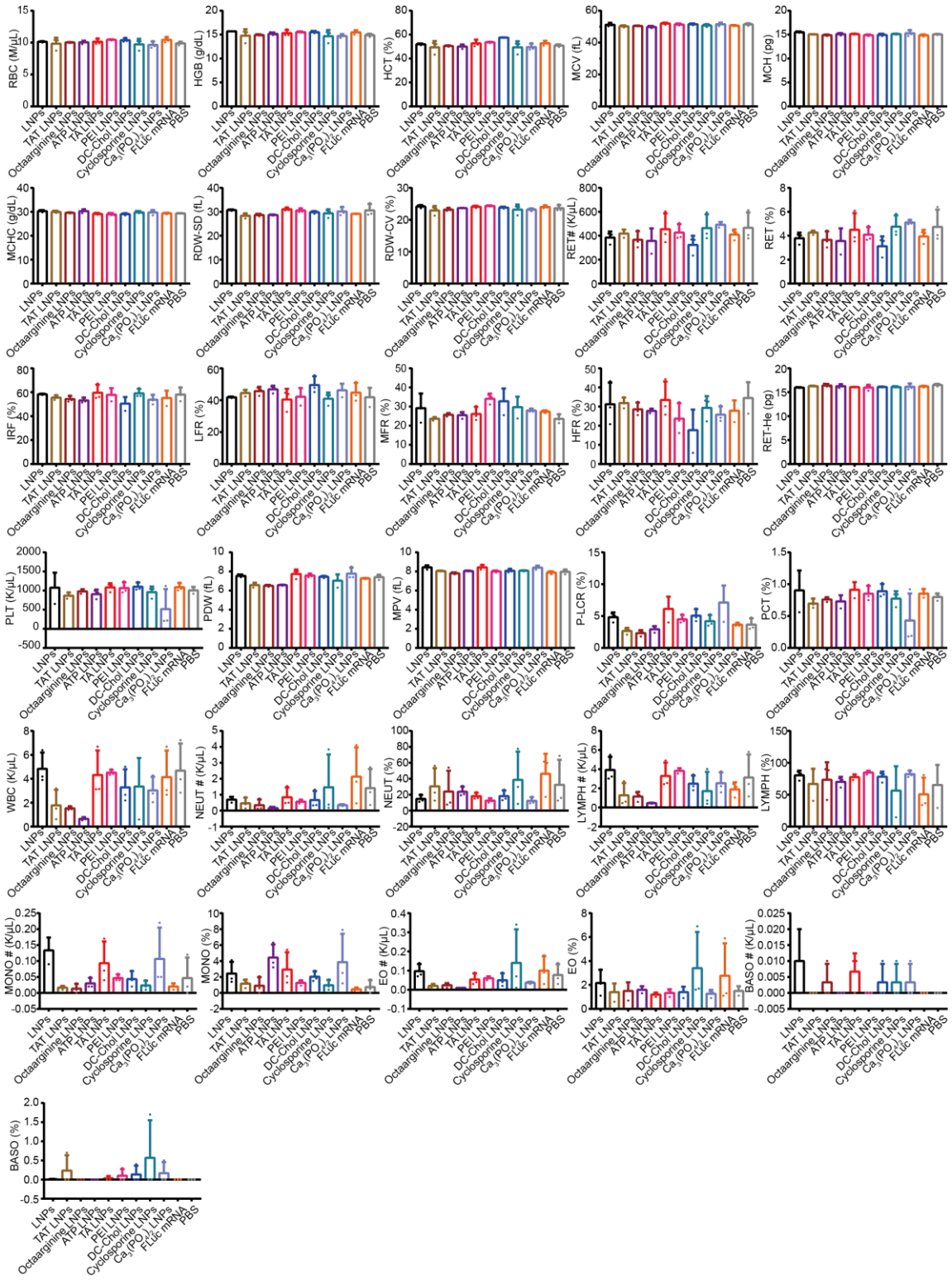
Supplementary Fig.9. After 24 h, fluorescence images of mRNA DiD-LNPs to indicate the biodistribution ex vivo (n = 3) for each group via intravenous (I.V.) administration are shown. Mice injected with naked FLuc mRNA and only PBS served as controls.



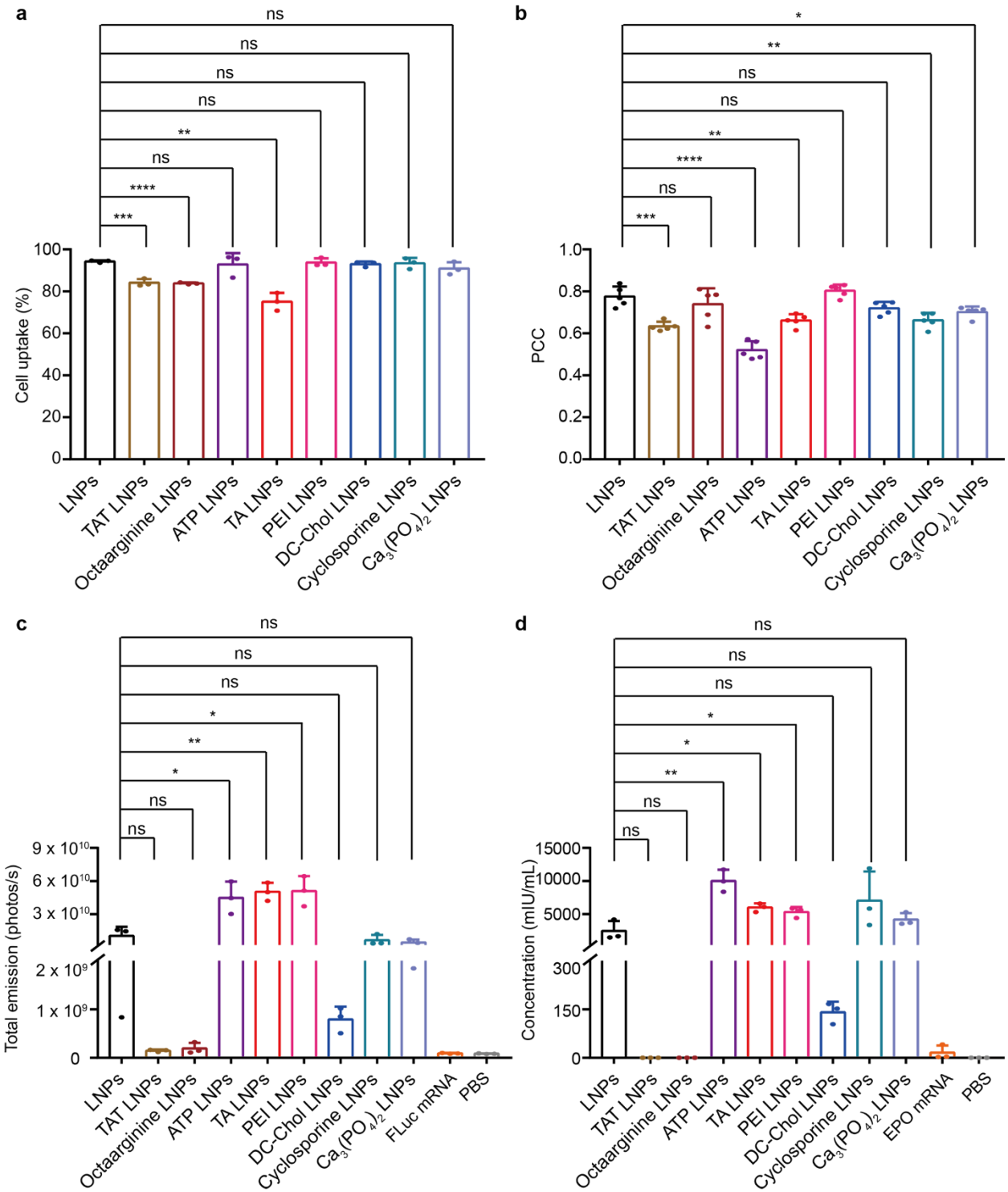
Supplementary Fig.10. (a), After 24 h, bioluminescence images of mRNA Moderna LNPs ex vivo (n = 3) for each group via I.V. administration are shown. Mice injected with naked FLuc mRNA and only PBS served as controls. **(b)**, After 24 h, fluorescence images of mRNA DiD-Moderna LNPs to indicate the biodistribution ex vivo (n = 3) for each group via intravenous (I.V.) administration are shown.



Supplementary Fig.11. Percent weight retention results. 24 h after respective intravenous dose of FLuc mRNA LNPs into mice (Data are shown for triplicates).



Supplementary Fig.12. Hematological tests testing CBC result of FLuc mRNA LNPs. Mice only injected with naked FLuc mRNA and PBS were used as controls (Data are shown for triplicates).



Supplementary Fig.13. Statistical analysis of data in Fig. 6c, 7c, 8c and 8e. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 and ns > 0.05 with 95% of confidence level from unpaired t-test (Data are shown for triplicates).

Supplementary Table S1. Size, PDI, zeta potential and mRNA encapsulation efficiency of Moderna LNPs

	Size (nm)	PDI	Zeta potential (mV)	mRNA Encapsulation Efficiency (%)
Moderna LNPs	155.1 ± 4.8	0.24 ± 0.02	-2.84 ± 5.40	56.1 ± 11.2

Supplementary Table S2. The N/P ratio of the LNPs

1. Molecular weight of SM-102: 710.182 µg/µmol
2. Amount of SM-102 used: 430 µg (has 1 amine group (N))
3. Amount of mRNA used: 40 µg (3 nmol of P / 1 µg of nucleic acid)
4. Moles of Nitrogen (N) in SM-102 (430 µg / 710.182 µg/µmol =0.61 µmol)
5. Moles of Phosphorus (P) in SM-102 (40 µg × 3 nmol/ 1 µg of nucleic acid =120 nmol = 0.12 µmol)
6. N:P Ratio = 0.61 µmol of nitrogen / 0.12 µmol of Phosphorus = 5.1