Liposomal Lipid Nanoparticles for Extrahepatic Delivery of mRNA

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Table S1. LNP's lipid composition, molar ratio of bilayer lipid to ionizable lipid ($R_{B/I}$), lipid molar ratio, N/P ratio and DLS measurement (black line: Intensity, blue line: Volume, red line: Number) for hydrodynamic diameter in both number means and Z-average (mean ± S.D., n = 3).

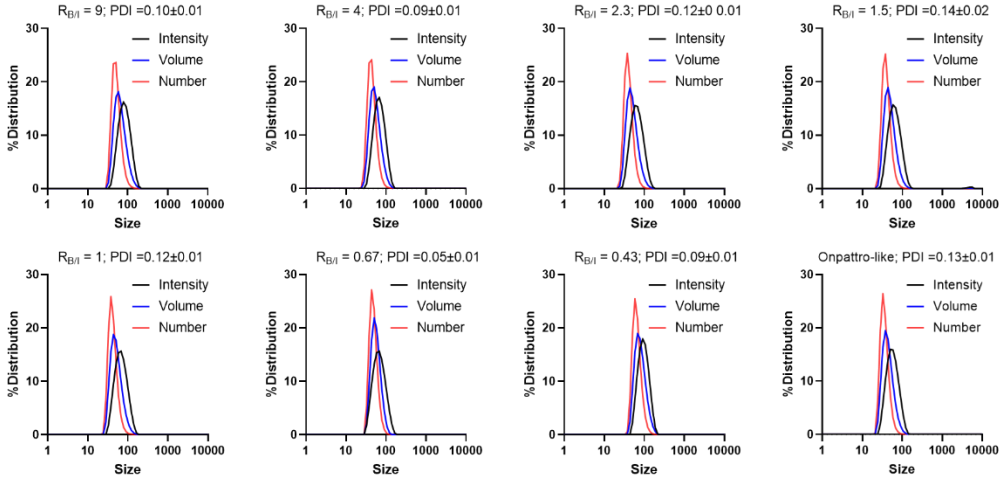
Liposomal LNPs

$R_{B/I}$	Nor-MC3	ESM	Cholesterol	PEG-lipid	NanoLuc mRNA (808 nt) N/P ratio	Hydrodynamic diameter [number mean] (nm)	Hydrodynamic diameter [Z-average] (nm)
9	10	45	45	1.5	6	53.0 ± 1.3	77.4 ± 0.8
4	20	40	40	1.5	6	45.4 ± 2.2	64.4 ± 0.2
2.3	30	35	35	1.5	6	41.4 ± 1.9	61.7 ± 0.4
1.5	40	30	30	1.5	6	40.2 ± 0.6	60.8 ± 0.6
1	50	25	25	1.5	6	42.8 ± 0.7	63.5 ± 0.4
0.67	60	20	20	1.5	6	49.2 ± 0.6	63.4 ± 0.2
0.43	70	15	15	1.5	6	66.4 ± 1.6	90.4 ± 0.7

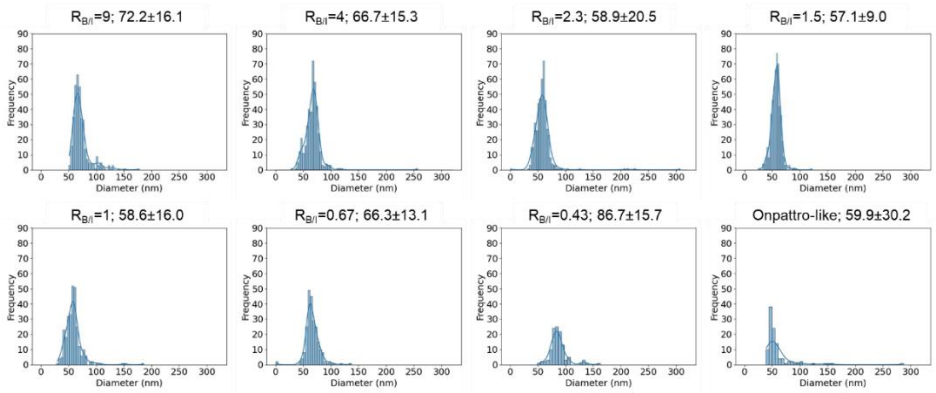
Onpattro-like

MC3	DSPC	Cholesterol	PEG-lipid	NanoLuc mRNA (808 nt) N/P ratio	Hydrodynamic diameter [number mean] (nm)	Hydrodynamic diameter [Z-average] (nm)
50	10	38.5	1.5	6	36.7 ± 0.5	53.9 ± 0.2

DLS quantification



Cryo-TEM quantification



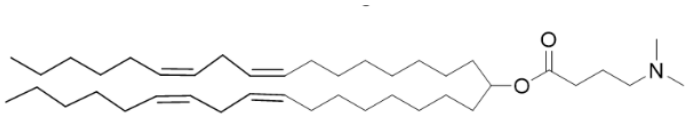
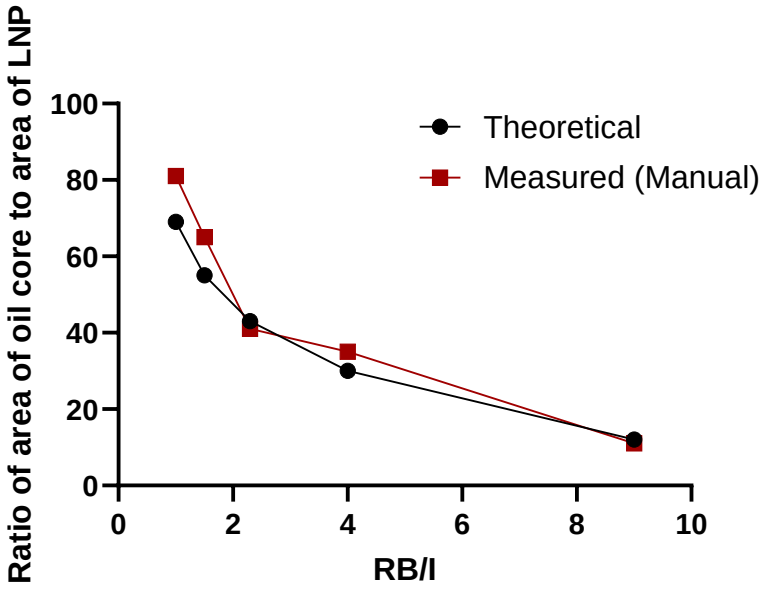


Fig S1. Chemical structure and characterization of (6Z,9Z,26Z,29Z)-Pentatriaconta-6,9,26,29-tetraen-18-yl 4-(dimethylamino) butanoate (nor-MC3).[1] The synthesis and characterization details can be found in the patent titled “MC3-type lipids and use thereof in the preparation of lipid nanoparticles”

Table S2. Ratio of oil core cross-sectional area to LNP cross-sectional area (among all LNP morphologies) as theoretically calculated [black line] and as measured (by manual annotation) [red line] from the Cryo-TEM analysis. The theoretical numbers assume the LNP contains a diameter of 50 nm, an area per molecule of ESM 0.5 nm^2, an area per molecule of cholesterol 0.3 nm^2, an area per molecule of ionizable lipid 0.3 nm^2, a lipid density of 0.9 mg/mL, and a membrane thickness of 5 nm.

$R_{B/I}$	Ratio of nor-MC3/ESM/Cholesterol	Theoretical ratio of oil core cross-sectional area to LNP cross-sectional area	Measured ratio of oil core cross-sectional area to LNP cross-sectional area
9	10/45/45	12	11
4	20/40/40	30	35
2.3	30/35/35	43	41
1.5	40/30/30	55	65
1	50/25/25	69	81



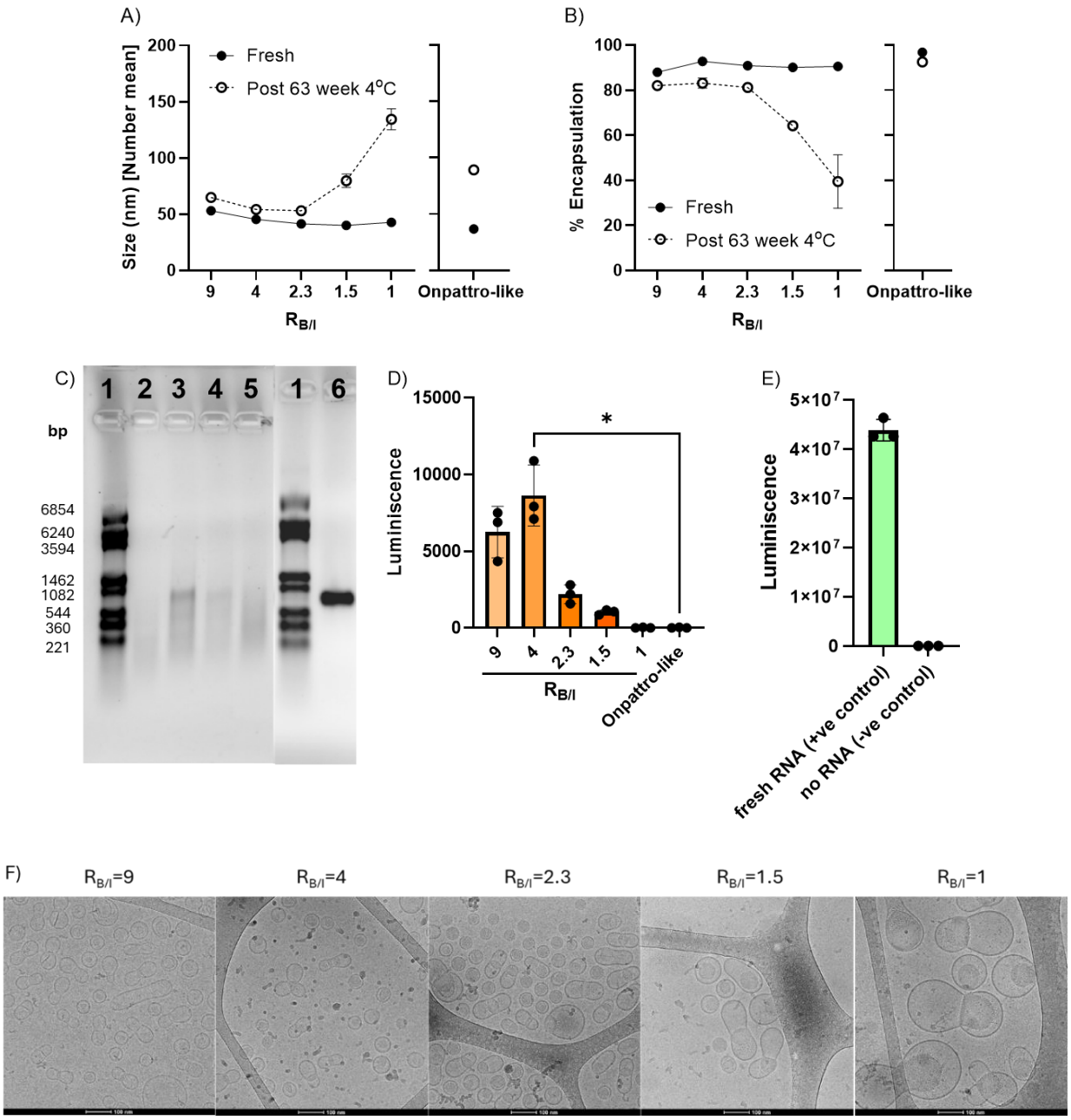


Fig S2. Stability of LNP encapsulated mRNA encoding NanoLuc after storage for 63 weeks at 4°C. LNP mRNA systems were formulated as described in Methods containing NanoLuc mRNA (808 nt; N/P=6) with a lipid composition of nor-MC3/ ESM: cholesterol/ PEG-DMG at varying ratios of bilayer lipid (ESM and equimolar cholesterol) to ionizable lipid ($R_{B/I}$). The Onpattro-like formulation consists of MC3/DSPC/cholesterol/PEG-DMG (50/10/38.5/1.5; mol/mol). A) Size in number means (nm) determined via DLS of LNP formulations at fresh (0 week) and 63 weeks. B) Encapsulation efficiency determined via RiboGreen assay of LNP formulations at fresh (0 week) and 63 weeks. C) RNA agarose gel of mRNA extracted from LNPs with varying $R_{B/I}$ (1: Ladder, 2: $R_{B/I}$:9, 3: $R_{B/I}$:4, 4: $R_{B/I}$:2.3, 5: $R_{B/I}$:1.5; 6: non-formulated NanoLuc mRNA, 250 ng/well). D) NanoLuc mRNA extracted liposomal LNP systems shows significantly higher translation competence in cell-free wheat germ lysate than mRNA extracted from Onpattro-like formulation (mean ± S.D., n = 3). Data were analyzed through a two-tailed unpaired t-test. ns = non-significant, *p < 0.05. The exact p-values can be found in the source data file. E) NanoLuc

mRNA shows higher translation competence in cell-free wheat germ lysate than mRNA extracted from LNP formulation (mean ± S.D., n = 3). F) Cryo-TEM images demonstrating the change of morphologies of LNP formulated under $R_{B/I}$ of 9-1. Micrographs are from one set of LNP samples.

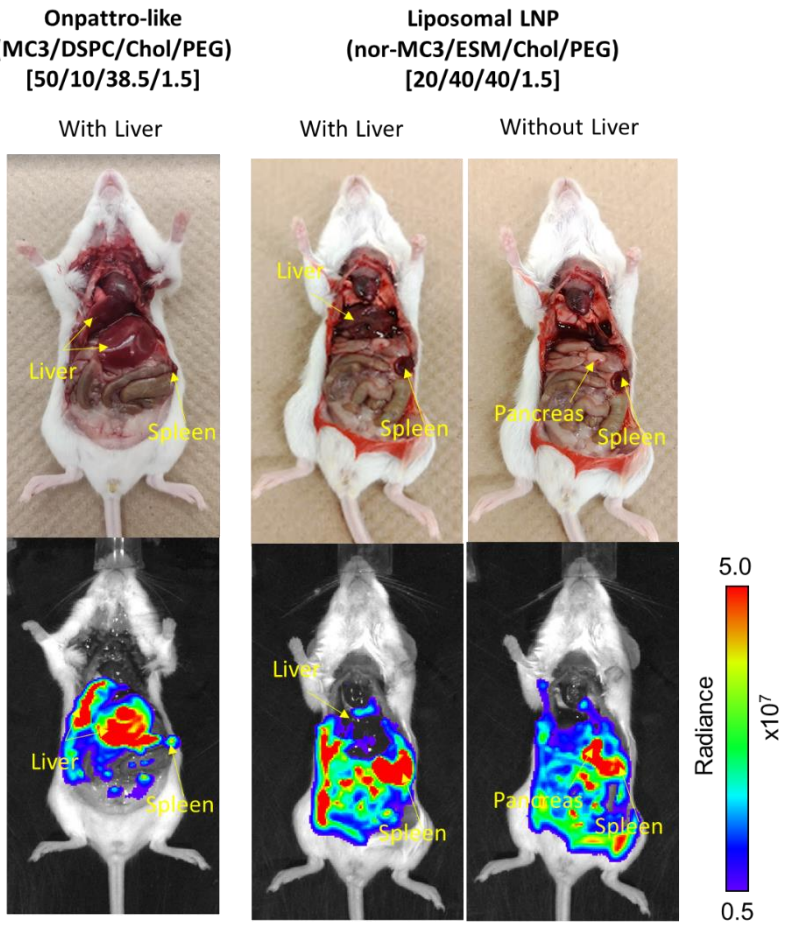


Fig S3. Liposomal LNP-NanoLuc shows extrahepatic delivery, and transfection in spleen and pancreas. In situ bright field and bioluminescence images of CD-1 mice i.v. injected with Onpattro-like and liposomal LNP ($R_{B/I}$=4) encapsulated with NanoLuc-mRNA at 0.5 mg/kg at 24 hpi.

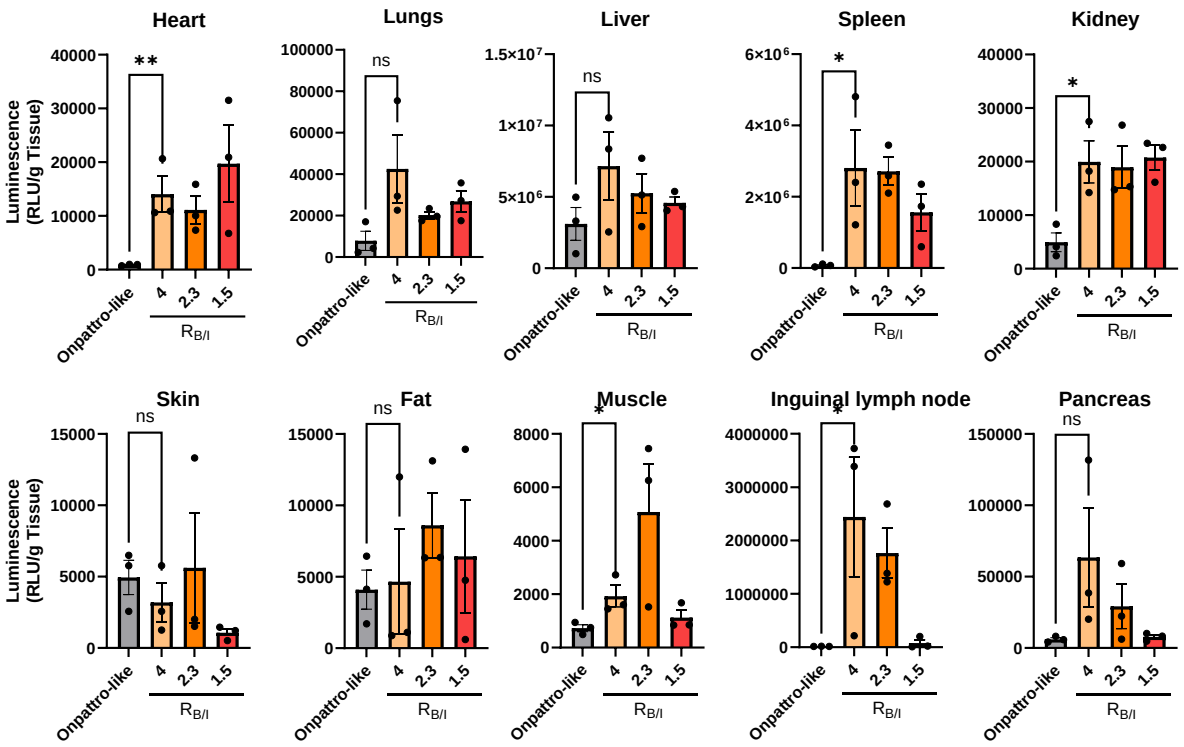


Fig S4. Bioluminescence signals of liposomal LNP-NanoLuc mRNA systems prepared with increasing $R_{B/I}$ through ex vivo luminescence measurement of homogenized tissue (across a wide range of tissues). Onpattro-like (MC3/DSPC/cholesterol/PEG-DMG, 50/10/38.5/1.5; gray bars) and liposomal LNPs ($R_{B/I}$=1.5-4; light orange to red bars) with N/P=6 was formulated in 25 mM NaOAc pH 4 buffers and were administered i.v. to CD-1 mice at an mRNA dose of 0.5 mg/kg. At 24 hours post injection the mice were sacrificed, organs or tissues removed, and ex vivo bioluminescence assayed by using Nano-Glo luciferase assay. (mean ± SEM, n = 3). Data were analyzed through a two-tailed unpaired t-test. ns = non-significant, *p < 0.05, and **p < 0.01. The exact p-values can be found in the source data file.

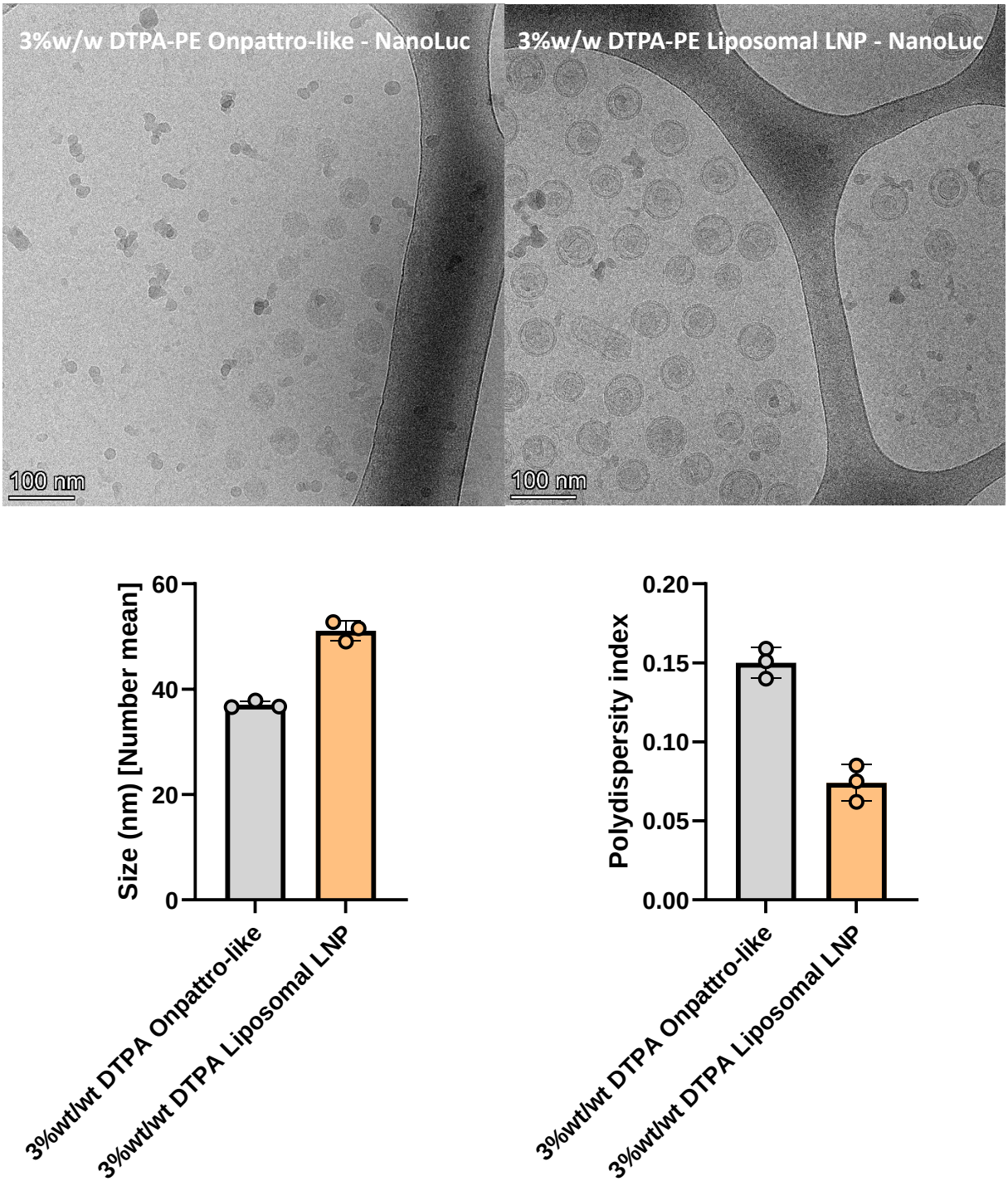
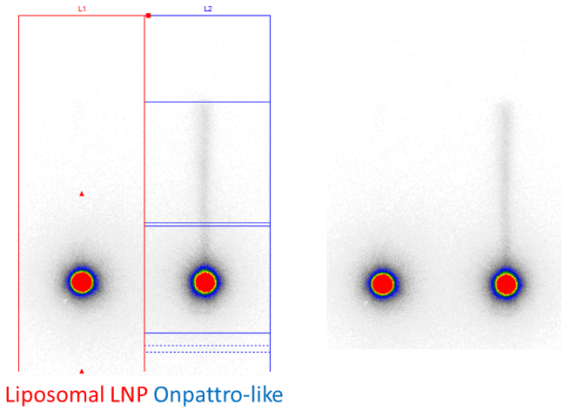
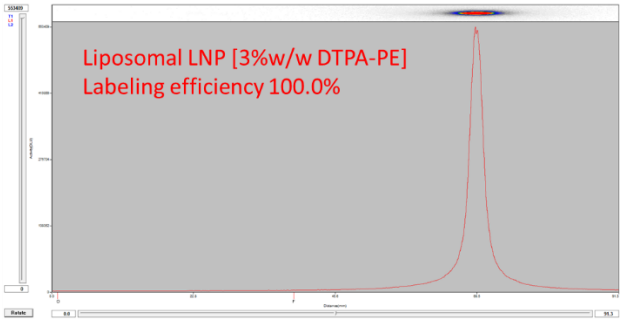
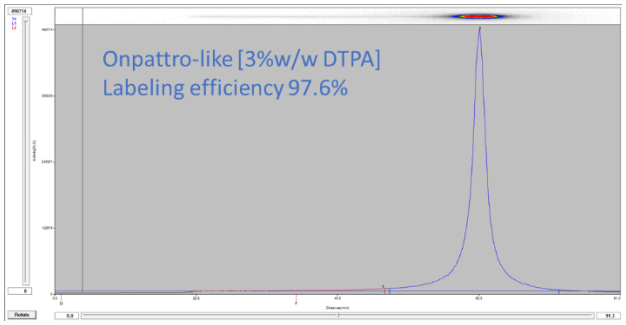


Fig S5. Representative cryo-TEM images of Onpattro-like [grey bar] and liposomal LNP ($R_{B/I}$: 4) containing 3% w/w DTPA-PE [orange bar] and encapsulated with NanoLuc encoded mRNA in PBS (mean ± S.D., n = 3). Micrograph has been reproduced twice.







Lane #1
Background Subtraction: Regions = 0.000 DLU /mm2

ID	Gross DLU	Background Subtract	Net DLU	Net % Sum	Net % Total Lane
Lane	28,158,725.5	0.0	28,158,725.5		100.0
UnRes	28,158,725.5	0.0	28,158,725.5		100.0

Lane #2
Background Subtraction: Regions = 2,255.129 DLU /mm2

ID	Gross DLU	Background Subtract	Net DLU	Net % Sum	Net % Total Lane
1 - Prof	2,755,974.7	2,267,010.6	488,964.1	2.4	2.4
2 - Prof	22,291,463.9	1,994,969.3	20,296,494.6	97.6	101.0
1-b - Prof	120,907.3				
Lane	26,759,695.5	6,665,011.1	20,094,684.4		100.0
UnRes	1,712,257.0	2,403,031.2	-690,774.3		-3.4

Fig S6. Representative phosphor images of ^{111}In-liposomal LNP ($R_{B/I}$: 4) and^{111}In-Onpattro-like containing 3% w/w DTPA-PE and encapsulated with NanoLuc encoded mRNA. The LNPs were developed through radio iTLC. Chelated ^{111}In- liposomal LNP and^{111}In-Onpattro-like remain at the baseline (R_f = 0). The radiochemical yield of ^{111}In-liposomal LNP and^{111}In-Onpattro-like was calculated as 100 and 97.6%, respectively.

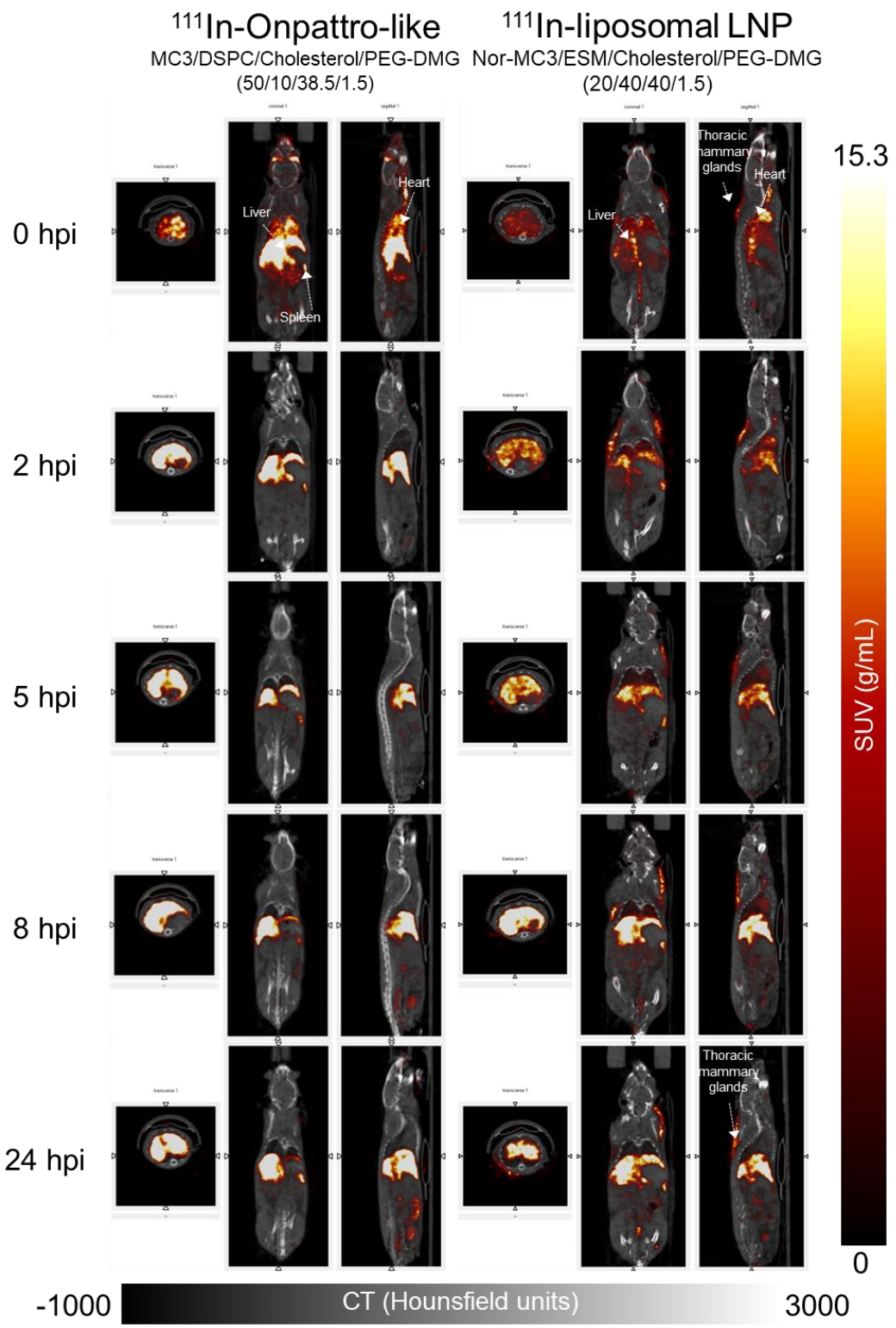


Fig S7. Representative sagittal, coronal, and transverse SPECT/CT reconstructed images of CD-1 female mouse intravenously injected with ^{111}In-Onpattro-like and ^{111}In-liposomal LNP ($R_{B/I}$: 4) encapsulated with NanoLuc encoded mRNA (~0.4-0.5 mCi/mouse- [^{111}In] and 15 µg, n = 3, ±SEM) at 24 h post-injection in CD-1 female mice.

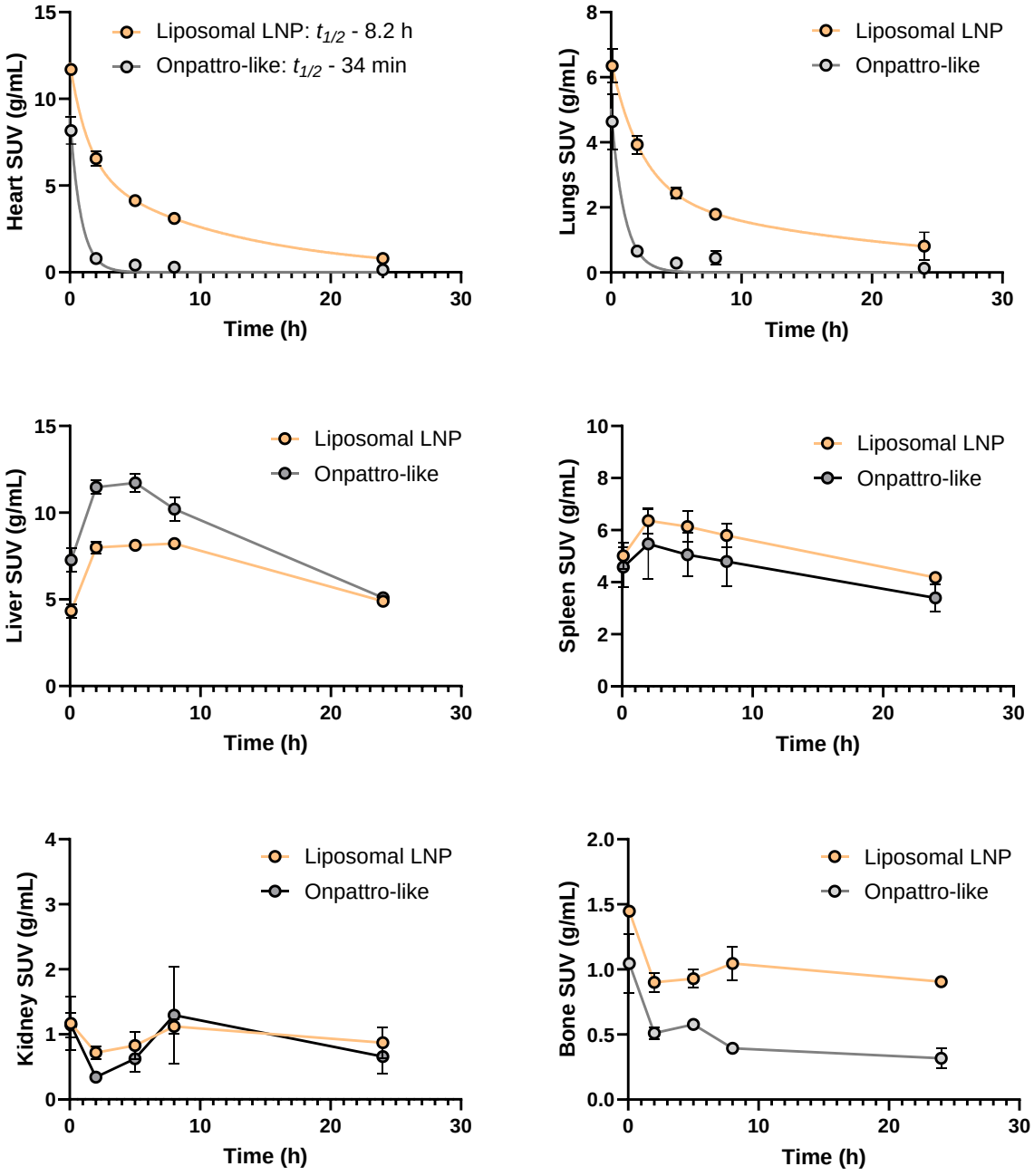


Fig S8. Standardized uptake values (SUV) of ^{111}In-Onpattro-like and ^{111}In-liposomal LNP ($R_{B/I}$: 4) encapsulated with NanoLuc encoded mRNA (~0.4-0.5 mCi/mouse- [^{111}In] and ~15 µg mRNA, n = 3, mean ± SEM) in heart, lungs, liver, spleen, kidney and bone at 0, 2, 5, 8, 24 h post-injection in CD-1 female mice. [Grey line = ^{111}In-Onpattro-like, Orange line = ^{111}In-liposomal LNP].

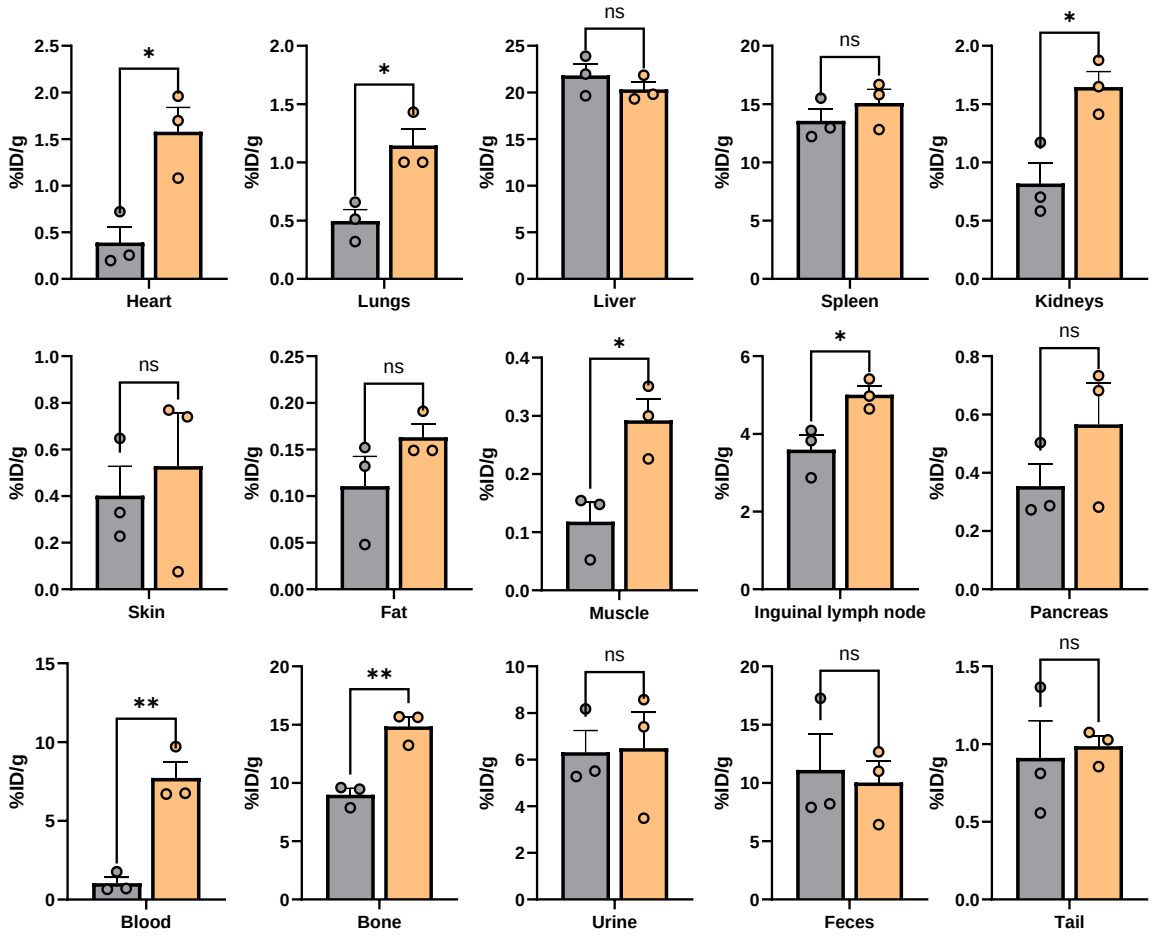


Fig S9. Biodistribution of intravenously injected ^{111}In-Onpattro-like and ^{111}In-liposomal LNP ($R_{B/I}$: 4) encapsulated with NanoLuc encoded mRNA (~0.4-0.5 mCi/mouse- [^{111}In] and ~15 µg mRNA, n = 3, ±SEM) at 24 h post-injection in CD-1 female mice. [Grey bar = ^{111}In-Onpattro-like, Orange bar = ^{111}In-liposomal LNP]. Ex vivo sample were measure through gamma-counting and have been decay corrected. Data were analyzed through a two-tailed unpaired t-test. ns = non-significant, *p < 0.05, and **p < 0.01. The exact p-values can be found in the source data file. Data were corrected for organs that could not be entirely removed (blood, muscle, bone) using literature organ weights for mice.[2]

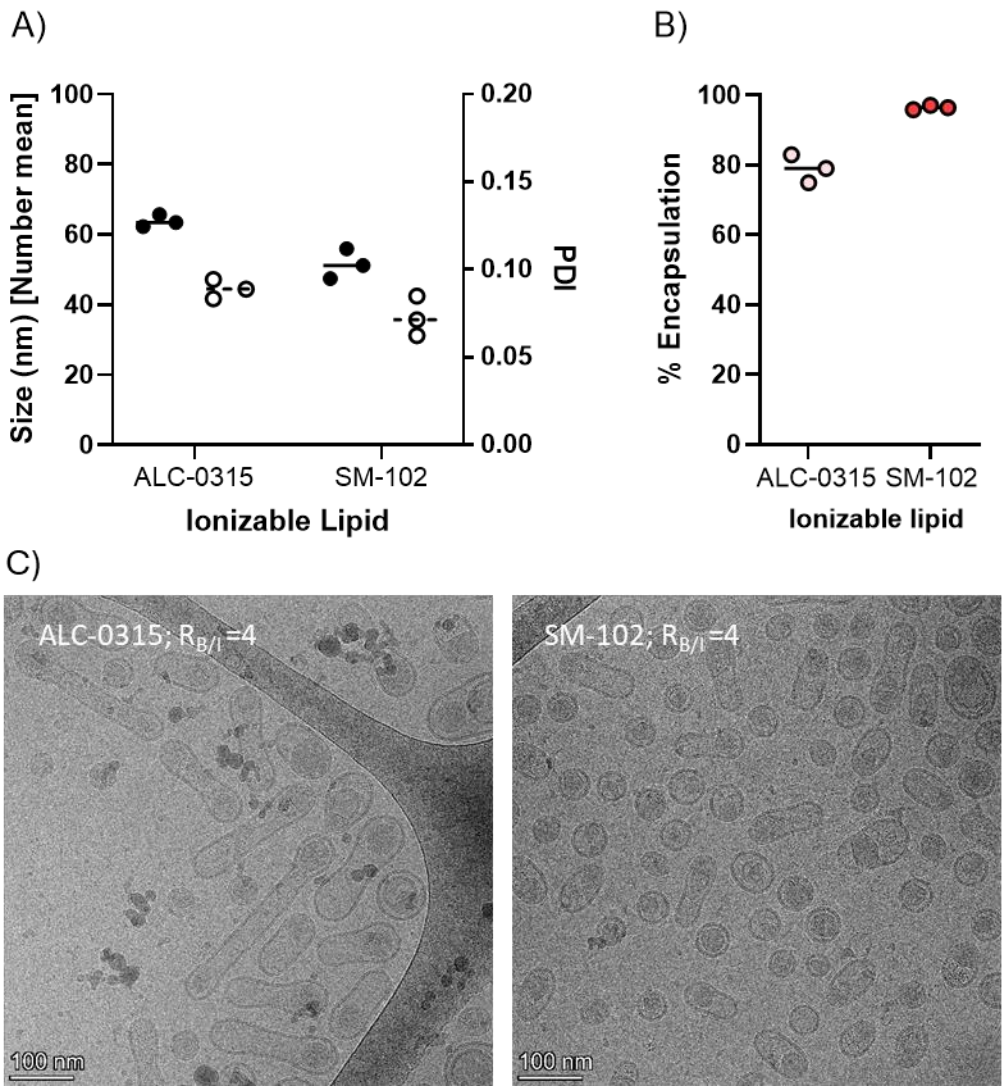


Fig S10. Liposomal LNP (ionizable lipid/ESM/cholesterol/ PEG-DMG; 20/40/40/1.5; $R_{B/I}$ = 4) formulated with ALC-0315 or SM-102 encapsulated with NanoLuc encoded mRNA at a N/P ratio of 6. A) Size in number means (nm) [filled dots] and PDI [hollow dots] determined via DLS (mean ± S.D., n = 3). B) Encapsulation efficiency determined via RiboGreen assay of ALC-0315 [pink dots] and SM-102 [red dots] liposomal LNP (mean ± S.D., n = 3). C) Cryo-TEM micrographs of LNP mRNA systems prepared using $R_{B/I}$= 4. Micrographs are from one set of LNP samples.

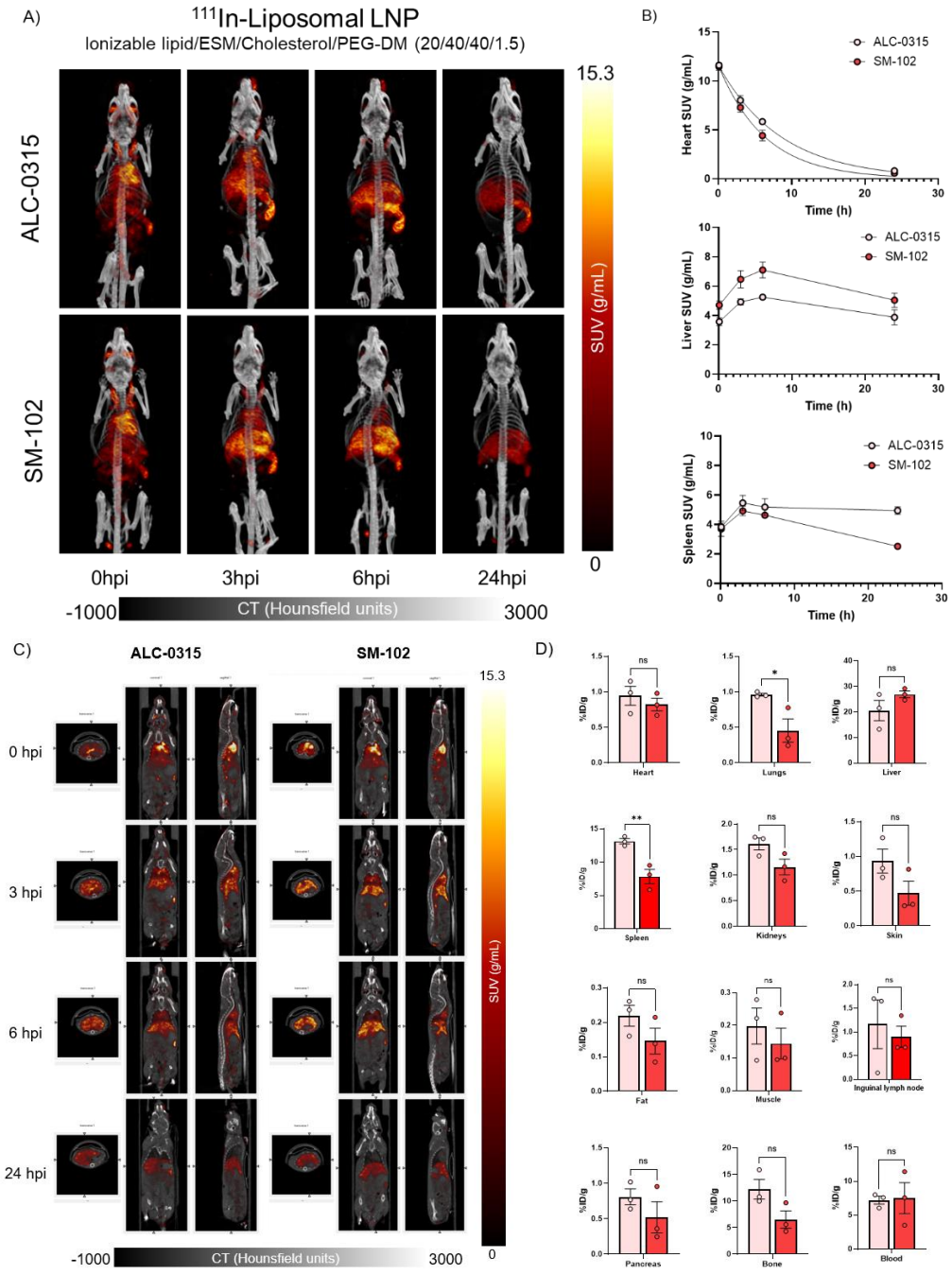


Fig S11. Radiopharmaceutical profile of ^{111}In-liposomal LNP ($R_{B/I}$: 4) formulated with either ALC-0315 or SM-102 encapsulated with NanoLuc encoded mRNA (~0.4-0.5 mCi/mouse- [^{111}In] and ~15 µg mRNA, n = 3, ±SEM) at 0, 3, 6, 24 h post-injection in CD-1 female mice. A) Representative 3D rendered SPECT/CT reconstructed images. B) Standardized uptake values (SUV) in heart, liver, spleen [Pink dots= ALC-0315, Red dots= SM-102]. C) Representative sagittal, coronal, and transverse SPECT/CT reconstructed images. D) Biodistribution measured by gamma counting at 24 h post-injection and has been decay corrected [Pink bar = ALC-0315, Red bar = SM-102]. Data were analyzed through a two-tailed unpaired t-test. ns = non-significant, *p < 0.05, and **p < 0.01. The exact p-values can be found in the source data file. Data were corrected for organs that could not be entirely removed (blood, muscle, bone) using literature organ weights for mice.[2]

Table S3. Biodistribution of ^{111}In-Onpattro-like (MC3/DSPC/cholesterol/ PEG-DMG; 50/10/38.5/1.5) and ^{111}In-liposomal LNP (ionizable lipid/ESM/cholesterol/ PEG-DMG; 20/40/40/1.5; $R_{B/I}$: 4) encapsulated with NanoLuc encoded mRNA in CD-1 female mice (%ID/g (mean) ± S.D) quantified by gamma counting at 24 hpi ((~0.4-0.5 mCi/mouse- [^{111}In] and ~15 µg mRNA, n = 3, ±SEM).

Ionizable lipid		**nor-MC3**	**ALC-0315**	**SM-102**
Organs	**^{111}In-Onpattro-like (%ID/g)**	**^{111}In-liposomal LNP ($R_{B/I}$=4) (%ID/g)**		
Blood	1.04±0.36	7.72±0.99	7.21±0.59	7.50±3.95
Urine	6.32±0.93	6.49±1.54	3.61±0.58	2.31±0.90
Feces	11.12±3.07	10.03±1.87	3.02±0.30	2.99±1.48
Tail	0.91±0.24	0.99±0.07	0.75±0.09	1.22±0.59
Bone	8.98±0.56	14.85±0.81	12.21±1.84	6.51±2.81
Spleen	13.57±1.00	15.10±1.17	13.13±0.41	7.83±1.85
Pancreas	0.35±0.07	0.57±0.14	0.81±0.11	0.52±0.38
Kidneys	0.82±0.18	1.65±0.13	1.61±0.12	1.16±0.27
Liver	21.83±1.23	20.32±0.78	20.53±3.96	26.90±2.28
Heart	0.39±0.17	1.58±0.26	0.94±0.13	0.82±0.16
Lungs	0.50±0.10	1.15±0.14	0.96±0.02	0.45±0.28
Fat	0.11±0.03	0.16±0.01	0.22±0.03	0.15±0.07
Muscle	0.12±0.03	0.29±0.04	0.20±0.06	0.14±0.08
Skin	0.40±0.13	0.53±0.23	0.93±0.17	0.47±0.30
Inguinal LN	3.60±0.37	5.01±0.22	1.16±0.51	0.90±0.39

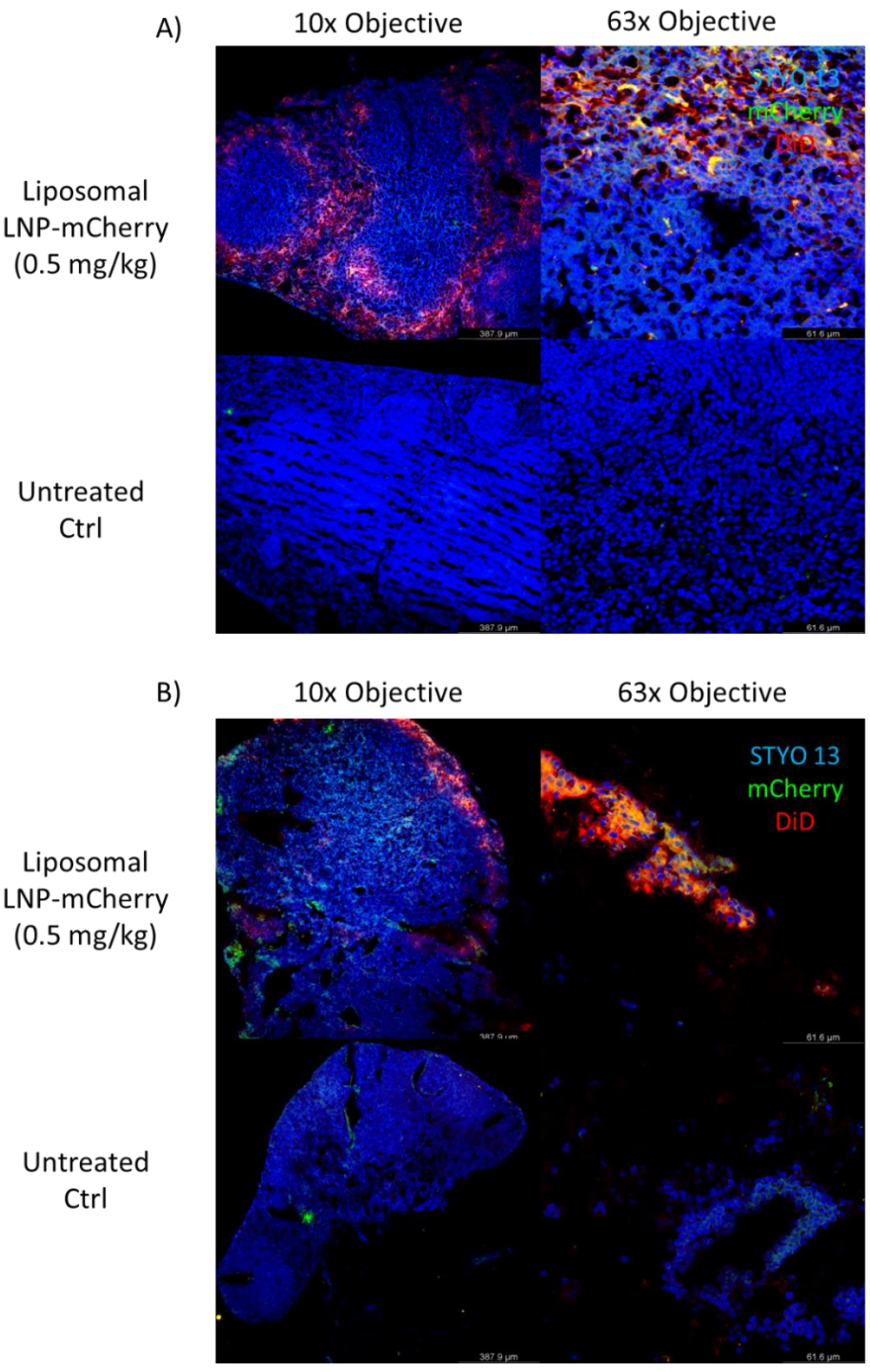


Fig S12. Fluorescence microscopy images of cryosection tissues from CD-1 female mice that were intravenously injected with liposomal LNP (nor-MC3/ ESM: cholesterol/ PEG-DMG; 20/40:40/1.5; $R_{B/I}$=4) containing mCherry mRNA (0.5 mg mRNA /kg) and sectioned at 24 h post injection. Microscopy experiment was repeated in three individual mice. The fluorescence channel is as follows: mCherry (green), and LNP labelled with DiD-C18 (red) and stained with STYO 13 (blue). A) For spleen, both mCherry and DiD-C18 signal were located the white pulp marginal zone and not at the geminal centre. B) For the inguinal lymph node, both signals are located at the peripheral of the lymph node at region such as the subcapsular and medullary sinus.

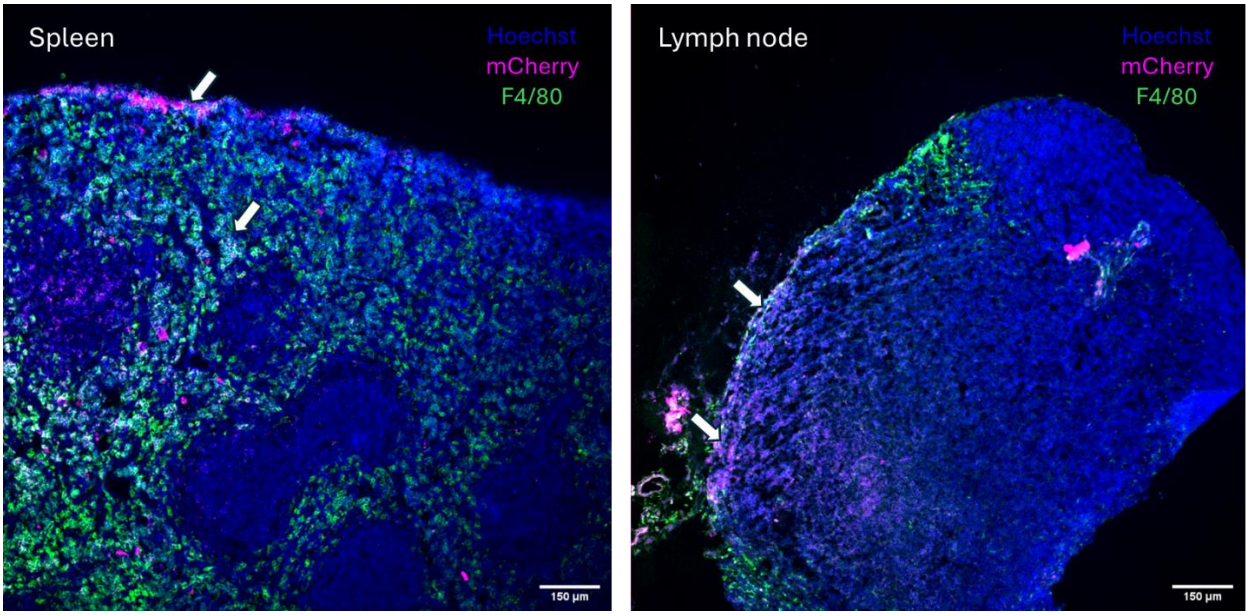


Fig S13. Immunohistology of cryosection spleen and lymph node tissues from CD-1 female mice that were intravenously injected with liposomal LNP (nor-MC3/ ESM: cholesterol/ PEG-DMG; 20/40:40/1.5; $R_{B/I}$=4) containing mCherry mRNA (0.5 mg mRNA /kg) and sectioned at 24 h post injection. Microscopy experiment was repeated in three individual mice. Tissues were directly stained with α-mF4/80 Alexa Fluor 488 Rat IgG2a clone:BM8 (AbLab) post sectioning.

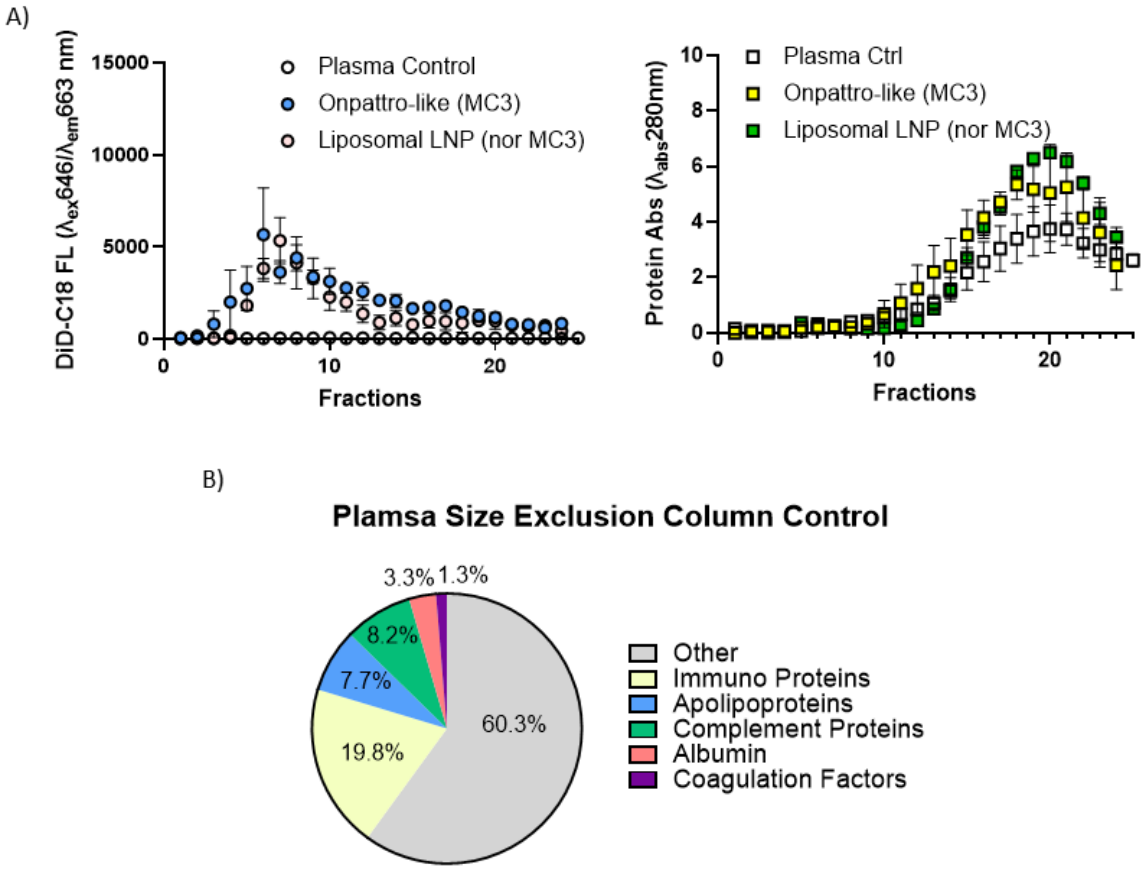


Fig S14. A) Fractions collected through size exclusion column chromatography for Plasma Control (n=4, ±SEM; hollow dots), Onpattro-like (MC3/DSPC/cholesterol/ PEG-DMG; 50/10/38.5/1.5; blue dots) and Liposomal LNP (nor-MC3/ESM/cholesterol/ PEG-DMG; 20/40/40/1.5; $R_{B/I}$: 4; pink dots) (n=3, ±SEM). Total protein measured from BCA assay and total lipid calculated from DiD-C18 absorbance measurement ($\lambda_{abs.}$ 649 nm) [Plasma Control – hollow squares; Onpattro-like – yellow squares; Liposomal LNP – green squares]. The DiD-C18 concentration was calculated with a molar extinction coefficient of 260 000 cm^{-1} M^{-1} and total lipid content was further calculated by DiD wt% of 0.8 mol%. B) The average abundance of proteins with distinct biological functions in the plasma control that has been collected after size exclusion column chromatography.

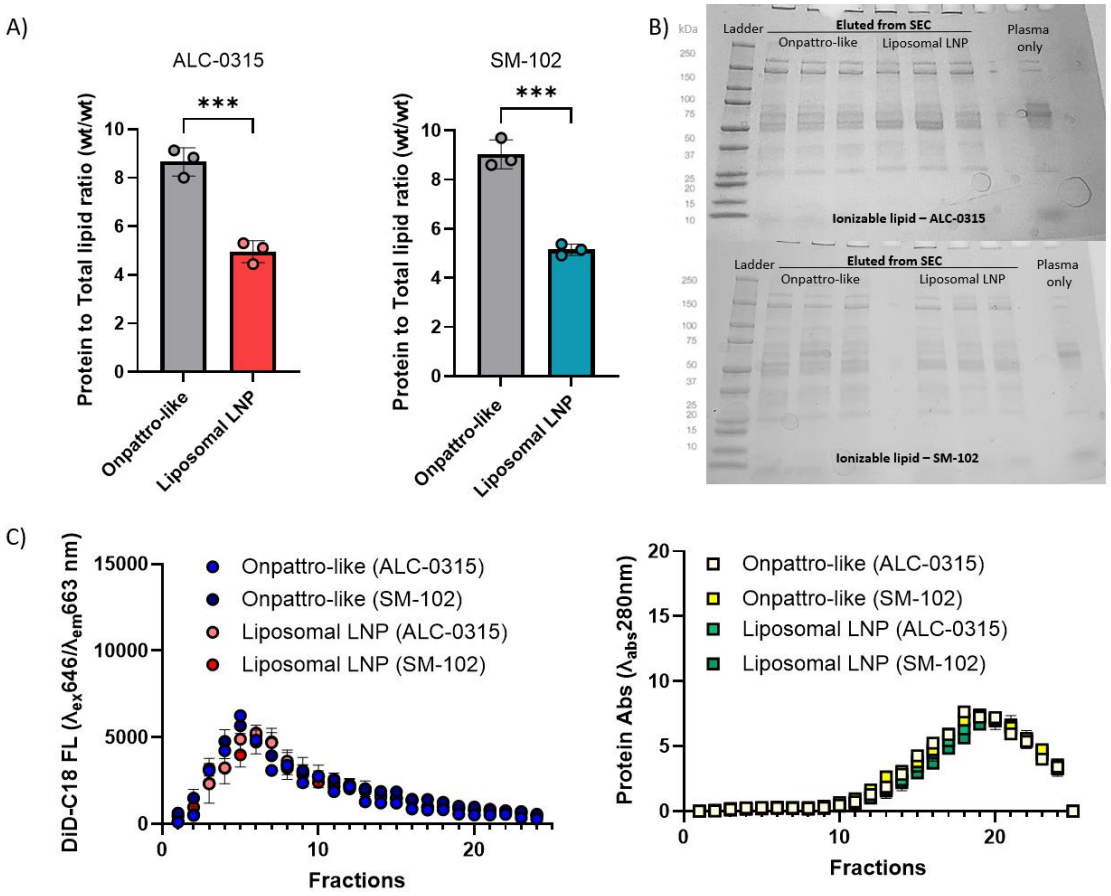


Fig S15. Liposomal LNP (either ALC-0315 or SM-102/ ESM: cholesterol/ PEG-DMG; 20/40:40/1.5; $R_{B/I}$=4; red or blue bars) and LNP with the Onpattro-like composition (either ALC-0315 or SM-102/DSPC/cholesterol/DMG-PEG: 50/10/38.5/1.5; gray bars) formulated with different ionizable lipids, N/P ratio of 6, were formulated, incubated with plasma and isolated as indicated in Methods. A) Protein to lipid ratio (wt/wt, µg/µg), total protein measured from a BCA assay, total lipid calculated from DiD-C18 absorbance measurement as indicated in Methods. Data were analyzed through a two-tailed unpaired t-test. ns = non-significant, ***$p < 0.001$. The exact p-values can be found in the source data file. B) SDS–PAGE of the plasma proteins adsorbed to the surface of Onpattro-like and liposomal LNP at 1 hour post incubation. The plasma protein profile is also shown. C) Fraction collected through size exclusion column chromatography for Onpattro-like and Liposomal LNP (n=3, ± SEM). Total protein measured from BCA assay and total lipid calculated from DiD-C18 absorbance measurement ($\lambda_{abs.}$ 649 nm) [Onpattro-like – light or dark blue dots; Liposomal LNP – light pink or dark pink dots]. The DiD-C18 concentration was calculated with a molar extinction coefficient of 260 000 cm^{-1} M^{-1} and total lipid content was further calculated by DiD wt% of 0.8 mol%. [Onpattro-like – cream or yellow squares; Liposomal LNP – light green or dark green squares].

Table S4. The proportions of proteins with distinct biological functions in the protein coronas of LNP mRNA systems with the Onpattro-like lipid composition (MC3/DSPC/cholesterol/ PEG-DMG; 50/10/38.5/1.5) as compared to the liposomal LNP (nor-MC3/ESM/cholesterol/ PEG-DMG; 20/40/40/1.5; $R_{B/I}$: 4) and plasma control. LNPs and proteins were incubated in gender pooled mouse plasma and separated by size exclusion chromatography, performed in triplicate (n=3 ± S.D.)

Protein categories	**Onpattro-like (%Abundance)**	**Liposomal LNP ($R_{B/I=4}$) (%Abundance)**	**Plasma Control**
Other Proteins	42.6±0.7%	43.9±0.1%	60.0±0.2%
Immuno Proteins	40.6±1.4%	36.4±2.7%	19.6±0.2%
Apolipoproteins	7.6±1.2%	11.0±2.4%	7.6±0.9%
Complement Proteins	4.6±0.2%	4.5±0.3%	8.2±0.1%
Albumin	3.7±0.5%	3.5±0.4%	3.2±0.3%
Coagulation Factors	0.9±0.1%	0.8±0.03%	1.3±0.1%

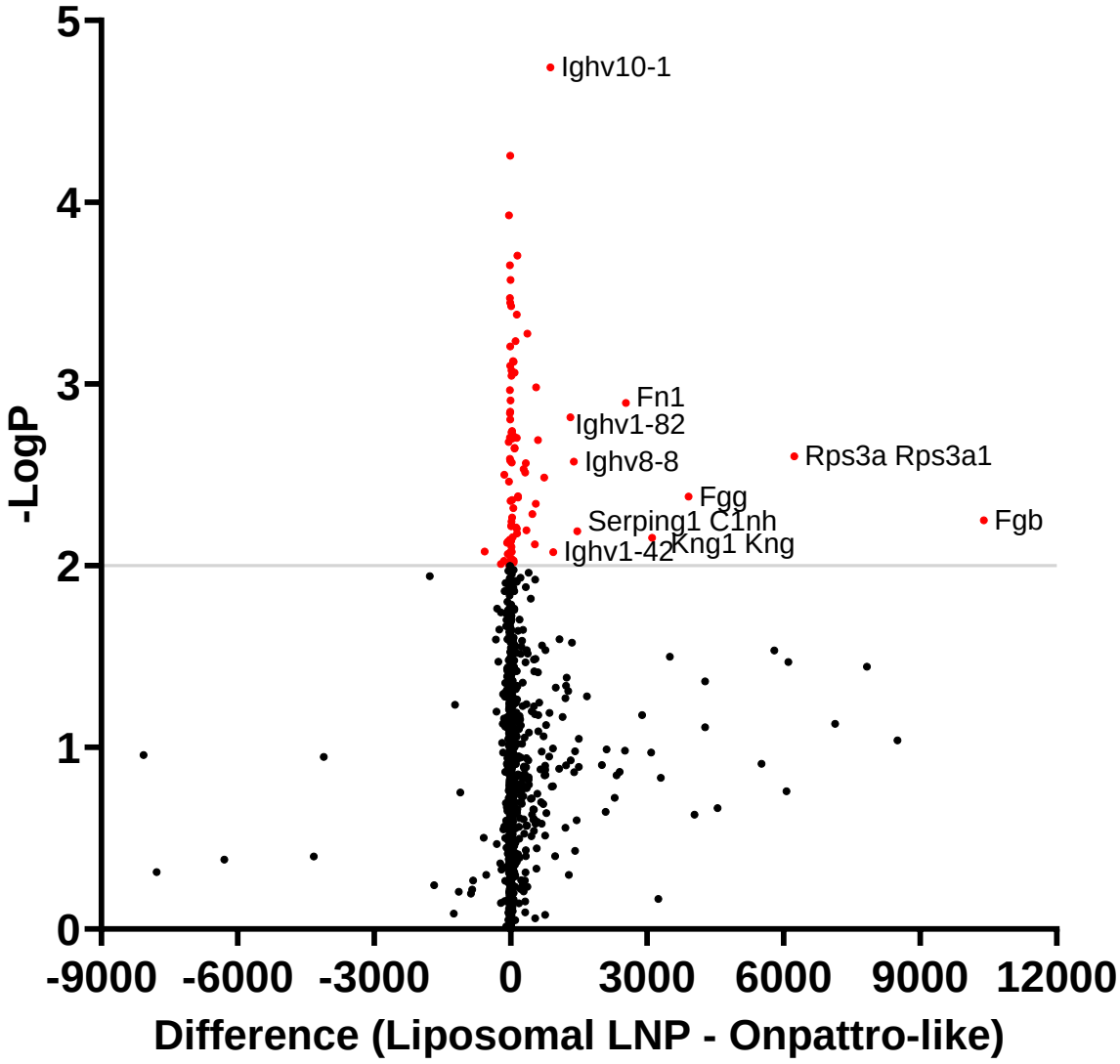


Fig S16 Volcano plot representing the statistical difference in protein content between liposomal LNP (nor-MC3/ ESM: cholesterol/ PEG-DMG; 20/40:40/1.5; $R_{B/I}$=4) and LNP with the Onpattro-like composition (MC3/DSPC/cholesterol/DMG-PEG: 50/10/38.5/1.5) where both encapsulated with NanoLuc encoded mRNA. -LogP represents the statistical difference (P=0.01) between the two formulations (n=3), with the highest values indicating that proteins (red dots) are more enriched in the Onpattro-like samples while the lowest values indicate that proteins are more enriched in the liposomal LNP ($R_{B/I}$: 4) samples. Values around zero indicate no difference.

Supplementary References

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pEJ2120 (BetaglobinUTR - Nluc - Betaglobin UTR)

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Supplementary Document: Theoretical calculation of ratio of solid core area to LNP area

Liposomal Lipid Nanoparticles for Extrahepatic Delivery of mRNA

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Proportion of solid core as a function of ratio of bilayer lipid to ionizable lipid		
Ratio of bilayer lipid to ionizable lipid	4	
R3 (nm)	20	
Area/molecule helper lipid (ESM) nm2	0.5	
Area/molecule cholesterol nm2	0.3	
Area/molecule ionizable lipid in positively charged form nm2	0.8	
Thickness of bilayer nm	5	
Number of helper lipids per LNP		9.82E+03
Number of ionizable lipids per LNP		2.45E+03
Density of cationic lipid (oil droplet) (gm/ml)	0.9	
MW of cationic lipids (KC2)	642	
Volume of cationic lipids per LNP (nm3)		2.91E+03
Radius of cationic lipid (oil droplet) nm		1.39E+01
Ratio of area of solid core to area of LNP		4.80E-01
Take into account limited solubility of ionizable lipid in lipid bilayer		
Solubility of ionizable lipid in lipid bilayer (mol/mol)	0.1	
Number of ionizable lipids in bilayer		9.82E+02
Number of ionizable lipids in oil droplet		1.47E+03
Volume of oil droplet		1.74E+03
Radius of oil droplet		1.25E+01
Ratio of area of solid core to area of LNP		3.89E-01