nature portfolio

Peer Review File

Liposomal Lipid Nanoparticles for Extrahepatic Delivery of mRNA

Corresponding Author: Dr Miffy Hok Yan Cheng

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, Cheng et al. present a rational design of LNP formulations with high proportions of bilayer-forming lipids, achieving improved mRNA encapsulation, enhanced transfection potency, and extended circulation times compared to the benchmark Onpattro in vivo. Crucially, this approach enabled efficient mRNA transfection in extrahepatic tissues, addressing a key challenge in the field. The study's findings expand the potential applications of LNP-based gene therapies beyond hepatic targets, opening new avenues for targeted mRNA delivery across various tissues. However, the main concern is whether the formulation strategy reported in this work can be extended to other ionizable lipids. Additional issues should also be addressed before this work is considered for publication, including clarity in text and figures and the need for additional controls to support the claims.
1. The authors studied a formulation using MC3, an ionizable lipid developed for siRNA delivery rather than mRNA delivery. It is essential to investigate whether this formulation strategy can increase the circulation time of LNPs with other ionizable lipids such as SM102 and ALC0315, which are widely used for mRNA delivery. This is a key weakness of the work.
2. The ESM-cholesterol formulation is not new. The authors' previous work has shown that extended circulation lifetimes can be achieved for liposomes composed of DSPC or sphingomyelin with approximately equimolar levels of cholesterol. It is essential to compare these formulations (DSPC vs. ESM) to emphasize the long-circulating property afforded by the ESM-cholesterol formulation in this work.
3. Please label the organs in Figure 2B, which currently looks confusing. Also, clarify the location in mice where muscle tissues were collected.
4. The 3D rendered SPECT/CT imaging used to study biodistribution (Figure 2E) does not show significant differences in extrahepatic delivery compared to Onpattro. Please revise this figure and include a statistical comparison of %ID/g in each organ.
5. The authors claim that reduced plasma protein adsorption is attributed to the ESM-cholesterol liposomal formulation. To substantiate this, LNPs with other ionizable lipids (SM102, ALC0315) should be investigated in parallel to demonstrate whether the ESM-cholesterol liposomal formulation can generally reduce plasma protein adsorption.
6. Some helper lipids, such as DOPE, are known to increase mRNA transfection at the cost of stability. ESM may also lead to less stability compared to DSPC. A three-month stability study should be added.

Minor Issues
1. In Figure S9, some green and red signals are visible in the untreated control group. Please explain this.
2. In the liposomal LNP system (Figure 1A), why is the mRNA located in the aqueous phase of liposomal LNPs rather than in the ionizable solid core, as seen in typical LNPs?
3. For the SPECT/CT reconstructed images (Figure 2D and Figure S6), it is recommended to use the same SUV range bar to facilitate the comparison of signal strengths between groups. Additionally, the positions of some organs, such as kidneys, lungs, and bones, are difficult to discern in these figures. Thus, how can the signal strength of these organs be calculated accurately based on the figures?
4. There are many typographical errors in the manuscript; please revise them. For example: Line 109: "we have shown that that the proportion of LNP that are solid core can be modelled by assuming that the triolein or (neutral) ionizable lipid exists in a hydrophobic environment surrounded by a lipid monolayer with a lipid bilayer protruding from it."
Line 471: "Fluorescnece imaging and Immunofluorescence imaging."

Reviewer #2

(Remarks to the Author)

Dear Editor,

Overall, the manuscript describes a clever utilization of sphingomyelin (SM) in the presence of cholesterol(Chol) within standard 4-component LNPs and how tunning the ratios between Bilayer-forming components (SM+Chol) and a MC3 analogue can improve blood circulation time and extrahepatic transfection of a surrogate NanoLuc mRNA compared to Onpattro LNP composition.

The research borrows the concept from previous long-circulating liposomes which had demonstrated some levels of extrahepatic accumulation. Further, it builds well on their group's previous paper (Kulkarni et. al., Nanoscale, 2019), where they had explored the role of helper lipid and Chol in the formation and stability of siRNA-encapsulated LNPs.

Yet, the concepts of extrahepatic delivery of mRNA via combination of helper lipid and/or ratio alterations have been explored by other researchers such as Whitehead's group (LoPresti et.al., J Control Release, 2022) or Dahlman's group (Radmand et. al., NanoLetters, 2023) in different forms.Nevertheless, similar concepts. Therefore, while the study has come up with a few LNP conditions at N:P 6 and 1.5% PEG-lipid as long circulating carriers for NanoLuc mRNA, it perhaps lacks the novelty factor to be reported in Nature Communication. My humble recommendation for this report is to perhaps be considered for more specialized Journals.

In addition, the present manuscript could benefit from some revisions as below:

1. Unfortunately, I could not locate the LNP formulation section under "Materials and Methods" on how they have formulated mRNA(808 nt) with their different lipid blends. I would recommend including detailed enough LNP formulation method.

2. Unknowing about the formulation method, I find it interesting that Onpattro benchmark LNP with 808nt Luc mRNA has resulted in ~ 40nm(Fig 1C) particle size at N:P 6! Done several times by my group and several other credible researchers, Onpattro composition typically results in ~ 50-80 range with encapsulated siRNA or mRNA, using microfluidic micromixing followed by gentle dialysis. Given that the study reports the values as diameter, I would recommend doing n=5 independent formulations of Onpattro with and without their mRNA and report statistical analysis and PDI.

3. Given the role of i-lipid molar ratio on the resulting final particle size, I wonder wouldn't the authors anticipate larger changes in ultimate size as a function drastic variations in i-lipid ratio from 10%-70%? Although qualitative and representative, yet some of the TEM images in Fig 1E do not well support the reported DLS data, e.g. R b/l = 0.43. This strengthens the skepticism on the accuracy of the reported sizes.

4. Given that electrostatic binding of ionizable cationic lipid with mRNA backbone plays the key role in encapsulation of mRNA into the final LNP particle, how would the author explain ~85% EE for 10% i-lipid compared to 60% EE for 70% i-lipid? Also, wouldn't one expect to see change in EE as a function of change in i-lipid ratio between 10%-70%?(Fig 1B)

5. Could the authors explain why N:P 6? I would recommend a systematic supplementary data on explaining why the authors have come up with 6?

6. Since the suggested extrahepatic delivery approach has only been tested for Luc mRNA, would this be only specific to Luc mRNA or it could be applier for other types of mRNA regardless of translation to secreted or membrane-bound protein?

Reviewer #3

(Remarks to the Author)
The interesting study by Miffy et al. supports some of the previous findings from Pieter Cullis' laboratory. Namely, that increasing the proportion of the helper lipid component in LNP formulations lead to the formation of liposomal LNPs and that these LNP structures exhibit longer circulation time and higher transfection potency. In addition to provide supportive data to this LNP formulation concept, they also look into the potential mechanisms driving these attractive attributes.
Based on the mechanistic studies, including their novel warhead-model describing the interaction between the endosomal membrane and the liposomal LNPs, I believe this work could be suitable for publication in Nature Communication. However, some additions and adjustments to the manuscript, which are highlighted below are in my opinion needed before the manuscript is ready to be published.

Major comments:

1. Re. LNP composition: Previous studies by some of the authors have shown that B:I ratios of 4.2 (Kulkarni et al. ref. 17, based on DSPC and siRNA payload) and 2 (Chander et al. ref. 13, based on ESM and mRNA payload) give rise to solid core encapsulated liposome-like LNPs with increased circulation time and increased transfection potency in extrahepatic organs/tissues compared to their spherical solid LNP counterpart based on the standard B:I ratio of ~1.
Hence, the authors need to present some arguments for why it is relevant to evaluate a wide range of B:I ratios. They should

also provide some rational/context as to why they decided to use ESM and nor-MC3 (contains two methylene groups less in the lipid tails than the clinical relevant MC3) instead of the standard DSPC and MC3 helper and ionizable LNP lipids. Maybe it could also be relevant to discuss the high cholesterol content in the high B:I ratio LNP formulations. Further, why are the authors not using dihydrosphingomyelin (DHSM) instead of ESM taken into account the attractive pharmacokinetics of DHSM compared to ESM as highlighted in ref.9.

2. Re. LNP biodistribution/transfection potency: The authors should discuss the biodistribution/transfection data in more detail so that the reader understand whether the increased transfection potency of the liposomal LNPs in extrahepatic tissues is driven by an overall enhanced transfection potency across organs, a organ-selective transfection potency, or is (for the most part) driven by its extrahepatic delivery capabilities when compared to the “Onpattro” formulation.
Based on a visual look at Figure 2C, it looks like the liposomal LNP-mediated transfection in the liver is more than 5x10^6, while for “Onpattro” the corresponding value is 3x10^6 RLU/g, while their corresponding 111In-radiolabeled LNP biodistribution data show similar distribution to the liver for the two different LNP formulations. These data combined suggests a higher transfection potency of liposomal LNPs compared to “Onpattro” in the liver. Maybe the ratio of the luminescence values and the ID%/g numbers could be a useful measure in a discussion about the different transfection potency across torgans/issues. As part of this discussion, I suggest the authors to discuss why the biodistribution (Figure S8) and transfection level (Figure S3) are not highly correlated.
That said, I greatly appreciate the data shown in Figure 2C and E, as I find these data more quantitative than the IVIS imaging-based data (which are associated with large error bars as shown in Fig. S3). Maybe, the authors could also discuss the differences between the data/methods presented in Figure 2C and E and the IVIS-data presented in the Supplementary Material.

3. Re. the biomolecular corona (BCM) studies: From the BCM data presented in Figure S11A, which include large error bars, I find it difficult to understand the significance of the stated two-fold increased biomolecular corona on “Onpattro” relative to the liposomal LNPs. How each of the single technical replicates compare (protein per ~lipid(DID) content wise) with each other would be valuable information. Further, why are the retention volume of the void fraction different in the “Onpattro” samples compared to the liposomal LNP samples? Comparing the total protein content from the pooled 3-10 fractions across the “Onpattro”, Liposomal LNP, and bare plasma (no LNP) samples could also provide some understanding of the significance of the amount of plasma proteins that are shifted toward the void volume due to their adsorption to the LNPs. Maybe such comparison is difficult due to the different void retention volumes for each of the systems? Interestingly, the data presented in Figure S11A also highlights to some extent that the liposomal LNPs are more stable (in terms of particle/DiD integrity) in plasma than the “Onpattro” formulation according to the long and rather intense DiD fluorescence tail in the chromatograms of “Onpattro” compared to the liposomal LNP formulations. Maybe that is worth highlighting?

4. Re. the mechanism of endosomal escape: Regarding the “Mechanism of action for mRNA loading and intracellular delivery by liposomal LNPs” including Figure 4 A and B: Why do the authors not highlight the high degree of liposomal LNP aggregation at low pH observed in the cryo-TEM data in addition to the observed ‘warheads’. I would expect that the observed LNP aggregation supports the notion that liposomal LNPs are efficient in interacting with lipid membranes (in this case independent on the negatively charged endosomal lipids). Any complementary data that supports the warhead-mechanism would indeed increase the impact of this manuscript, as this part is in my mind novel.

5. Re. mRNA stability: In a previous study (https://onlinelibrary.wiley.com/doi/10.1002/adma.202303370) by some of the authors, they highlight that the stability of mRNA in bleb (bilayer/liposome-like) structures compared to mRNA encapsulated in spherical solid LNP structures resulted in an increased transfection potency. It would be highly relevant to discuss whether the increased transfection potency of the liposomal LNPs is also in part due to the increased stability of mRNA. I suggest the authors to evaluate where the mRNA is located (likely present in the large ‘bleb’/aqueous core in the liposomal LNP structure) and the stability of the mRNA in the liposomal and spherical solid LNPs like they do in the study highlighted above. Evaluating each of the different identified positive LNP attributes would increase our understanding of which and how much each attribute contributes to (in this case) the increased transfection potency.

6. Re. liposome control: Would it add value to the study to evaluate a liposome formulation based on 40 ESM:40 Chol:1.5 PEG-DMG in some of the studies (e.g., the biodistribution/half-life study) to highlight the ESM-Chol-PEG-lipid surface composition and liposomal nature of the liposomal LNPs? Or would the small proportion of the ionizable lipids at the liposomal LNP surface and potential size differences make such simple comparison useless?

Minor comments:

7. Lines 23-24: Not sure whether this statement is correct, as several research groups have shown that negatively and positively charged LNPs home to the spleen and lungs and I don't believe that these types of LNP formulations exhibit a long circulation time.

8. Lines 94-95: It is not clear what “1.5 mol ratio” (1.48) refers to in “maintaining the PEG-lipid content constant at 1.5 mol ratio”. Consider writing “1.5 molar percentage” or “1.5 mol%” instead.

9. Lines 117-118: “It may be noted that the oil droplet is located either in the centre of the LNP or associated with the membrane bilayer”. Please explain why the solid core is not always associated with the LNP membrane (likely a statistical argument). Keep in mind that the cryo-TEM provides two-dimensional projection images. Hence, the solid core observed in cryo-TEM in the center of the liposomal LNPs could in principle also be associated with the LNP membrane.

10. I am also missing a brief discussion about the data on “Onpattro” and the B:I = 1 data, as these LNP formulations are based on roughly similar lipid type ratio but differs with respect to helper (ESM vs DSPC) and ionizable (nor-MC3 vs MC3) lipid.

11. The following text in the Figure 1 caption is a bit confusing: “If it is assumed that the cholesterol and DSPC reside exclusively in monolayer or bilayer environments the RB/I for this
formulation is 1.03”. I assume that the B:I ratio is independent on where the lipids are localized in the LNP – it is a bulk lipid composition-based value.

12. Figure 1D caption “D) The proportion of LNP surface area occupied by solid core, electron dense, morphology vs. the total surface area of the LNP as determined from cryo-TEM micrographs.”. I believe according to the main text “The properties of the system containing bilayer lipid to ionizable lipid ratios RB/I=4 are of particular interest as a large proportion (~84%) exhibit a bilayer structure containing a solid core component that occupies approximately 30% of the interior (Fig. 1D).” that it is the percentage of LNPs with a visible aqueous compartment that is shown in Figure 1D (not the percentage of solid core area, which is shown in Table S2). Please consider exchanging the y-label text “% Morphology” with something that is more intuitively understandable.
Please also show in the Supplementary Material how you did the segmentation (oil versus total area) using one cryo-TEM image.

13. Define the “Onpattro” formulation at the beginning of the main text. Consider using the term "Onpattro-like" of ""Onpattro"" because Onpattro relies on a specific siRNA cargo and another type of PEG-lipid (PEG-C-DMG).

14. Line 154: Specify which lymph node(s) you analyzed.

15. Line 171-: Please provide some context to why the authors’ decided to focus on lymphoid organs when studying in vivo transfection on a cellular level.

16. Adjust typos, incl:
Line 37: "(i.v,)"
Line 471: “Fluorescnece imaging …”
Fig. S9: exchange “stained with STYO13” with “stained with SYTO 13”
Fig. S11B: “Plamsa Size Exclusion …”

17. Table S2 is very interesting! I expect it shows that the different lipids are localized differently in an LNP. Unfortunately, I cannot figure out how the authors calculated the theoretical numbers. The authors should provide more details about the calculation.

18. Re. the text “Table S1. Liposomal LNP’s lipid composition, molar ratio of bilayer lipid to ionizable lipid (RB/I), lipid molar ratio and N/P ratio.”: At low B:I ratios, the LNPs do not form liposomal LNPs. Hence, consider writing “LNPs’ lipid composition…” instead of “Liposomal LNP’s lipid composition…”.

19. Please provide some context to Fig. S2 about the different liver sizes and with and without liver in the figure caption.

20. You may find this new and interesting article (https://www.nature.com/articles/s41557-024-01584-z) relevant and worth discussing in your manuscript.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors addressed all of my questions satisfactorily.

Reviewer #2

(Remarks to the Author)
With respect to the novelty factor, author argues that previous reports(exemplified by me on the initial review comments) use cationic(DOTAP) or negatively charged lipids(PS) while this study uses uncharged lipid(ESM). Plus, author borrows from previous studies to generally claim cationic lipids reduce circulation besides activating compliments.

Yet, manipulation of helper lipid type (regardless of being uncharged or charged) and ratio with cationic and/or ionizable lipids have been reported in different case scenarios. Besides authors own group(Kulkarni et. al., Nanoscale, 2019), other reports have directly reported on how Sphingomyelin(SM)-modified lipid nanoparticles improve blood circulation as the main factor for extrahepatic delivery. For instance, Webb et al (Br J Cancer, 1995, Oct:72(4): 894-904) has already shown SM/Cholestrol offer higher blood circulation than DSPC/Cholestrol liposomes when injected I.V . Another example is the most recent work by Wang et. Al. (Nat. Comm. (2024) 15:2073), where they show Cholestrol/SM lipid nanoparticles improve blood circulation.

Based on above two direct published works and my previously exemplified indirect works, I believe this research is a derivative of those studies presented as an overall approach and does not justify the novelty factor for me. Perhaps, looking at PK and PD of such nanoparticles in large animals, e.g. NHPs, would add the novelty factor.

In terms of authors addressing my initial review comments:

1. Authors has kindly included the Materials & Methods.

2. For this comment, author has kindly provided the detailed data. Author tends to report the particle size in both number-weighted and Z-average (cumulative intensity-weighted) distribution values. Suggested by their Z-average data, Onpattro LNP with their RNA construct shows ~ 53.9nm which corroborates with my initial review suggestion and previous reports. I strongly suggest that the author replaces Fig 1c with Z-average sizes as their provided Cryo-EM statistical data in their revision for comment 3 matches significantly closer to their Z-average. As anticipated, given the assumptions for number-weighted calculations, their mean numbers are off in comparison to their Cryo-EM and Z-average data.

3. Author has kindly added some statistical Cry-EM data to shed light but they still have not explained why they do not observe the effect of i-lipid molar ratio.

4. Author offers a speculative argument which is a likelihood for the observed phenomenon.

5. Author has attempted single lipid/RNA ratio at N:P6, based on previous studies.

6. Author has not tried different mRNA constructs, yet study claims for all RNA delivery.

Taking all above into account, this research does not get my recommendation to be published in a Nature-family journal, rather a more specialized journal.

Reviewer #4

(Remarks to the Author)
The authors present a highly interesting manuscript covering liposome-like LNPs which show improved extrahepatic delivery of mRNA. As a fusion nanomedicine system (liposome-LNP) with compartmentalisation (i.e. a LNP bleb anchored to the inner leaflet of a liposome bilayer) the research will be of considerable interest to the LNP and liposomal nanomedicine communities. The results show promise in the system with improved in vitro transfection and a broader organ biodistribution profile at the 24 h time point compared to clinically approved LNP this is argued to be linked to the improved circulation time of the liposomal-LNPs due to the ESM/Chol liposome bilayer showing lower protein adsorption with emphasis on immunoproteins.

The authors provide extensive data around a number of key aspects (e.g. CT, IVIS, protein adsorption, nanomedicine structure dynamics) and adequately address the comments of Reviewers 1 to 3. There are areas where the authors could add further detail which is both scientifically interesting, does not require further experiments and would improve the manuscript clarity.

Comment 1: Reviewer 3's major comment 3 touches on the significance of the data presented in Fig.3, particularly in the context of Fig.3A. The authors present an argument that the ESM/Chol shows reduced protein adsorption (Fig.3A and prior literature) and highlight the reduced immuno protein levels on these bilayers compared to the monolayer surface of the onpattro LNP. However, Fig.3A only presents limited significance (P-value *) with the protein heat-map data giving many broad protein groups as almost identical between the 2 systems (e.g. other, complement, albumin, coagulation factors). Equally, the authors do not discuss the differences in apolipoprotein levels between the 2 systems.

The authors should add more detail here as they have good data (1000+ proteins) that would provide further clues as to why the ESM/Chol bilayer extends circulation time. If there is an argument that part of the mechanism of hepatocyte uptake of LNPs is linked to apolipoprotein coverage then why (given the proportional increase in apolipoprotein coverage for liposomal-LNPs) do liposomal-LNPs not get cleared quickly? I can see arguments associated with increased saturation of hepatocytes allows a larger % of liposomal-LNPs to circulate in the blood, arguments associated with actual number of these proteins/liposomal-LNP etc. but the authors should discuss this briefly. Equally, given the role of complement proteins in PEG immune responses it would be interesting to explore whether these were different between the 2 systems.

Comment 2: As a technical point, the main manuscript repeatedly does not define which lymph node the authors were analysing. In the supporting information we find in Fig. S9 that it is the inguinal lymph node. However, the authors do not discuss why the choice of this lymph node and what the biodistribution data means when this lymph node is significant (e.g. 2-10% of injected dose etc.). The authors should explain their decision here.

Comment 3: As another technical point, for many captions in the main manuscript and the supporting information it is not clear how many animals, organs, samples were used and in the case of organs or samples whether these come from the same or different mice. As I can see from the figures, the IVIS data shows 3 mice (Fig.2A) and Fig S11 mentions that the CT images represent 3 mice. This is on the low number of mice. I would expect for most exploratory biodistribution data to be use 5-6 mice per group. The authors should acknowledge potential limitations in biodistribution data if mice numbers are

low.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
The authors have adequately addressed my comments and the manuscript should be published in Nature Communications.

Reviewer #1 (Remarks to the Author):

In this manuscript, Cheng et al. present a rational design of LNP formulations with high proportions of bilayer-forming lipids, achieving improved mRNA encapsulation, enhanced transfection potency, and extended circulation times compared to the benchmark Onpattro in vivo. Crucially, this approach enabled efficient mRNA transfection in extrahepatic tissues, addressing a key challenge in the field. The study's findings expand the potential applications of LNP-based gene therapies beyond hepatic targets, opening new avenues for targeted mRNA delivery across various tissues. However, the main concern is whether the formulation strategy reported in this work can be extended to other ionizable lipids. Additional issues should also be addressed before this work is considered for publication, including clarity in text and figures and the need for additional controls to support the claims.

Response: We thank reviewer 1 for these comments, we provide a point-by-point response below:

1. The authors studied a formulation using MC3, an ionizable lipid developed for siRNA delivery rather than mRNA delivery. It is essential to investigate whether this formulation strategy can increase the circulation time of LNPs with other ionizable lipids such as SM102 and ALC0315, which are widely used for mRNA delivery. This is a key weakness of the work.

Response: To address the reviewer's comment, we have formulated with ALC-0315 and SM-102 using the $R_{B/I}$ ratio of 4. The size, PDI and encapsulation efficiency of ALC-0315 and SM-102 LNPs were comparable to the liposomal formulation ($R_{B/I}$=4) formulated with nor-MC3. The cryo-TEM images showed similar liposomal morphology, demonstrating that these different generation ionizable lipids can also be incorporated in the liposomal LNP system. Interestingly, cryo-TEM revealed that inclusion of ALC-0315 results in tubular LNP structure. This likely reflects the more fusogenic nature of ALC-0315 resulting in inter-particle fusion and larger structures. However liposomal LNP formulated with both ALC-0315 and SM-102 exhibited prolonged circulation lifetimes in vivo (comparable to liposomal LNP formulated with nor-MC3) as assayed using SPECT/CT imaging. ALC-0315. These data have been included as Fig. S10-11 and highlighted in the main text in the manuscript.

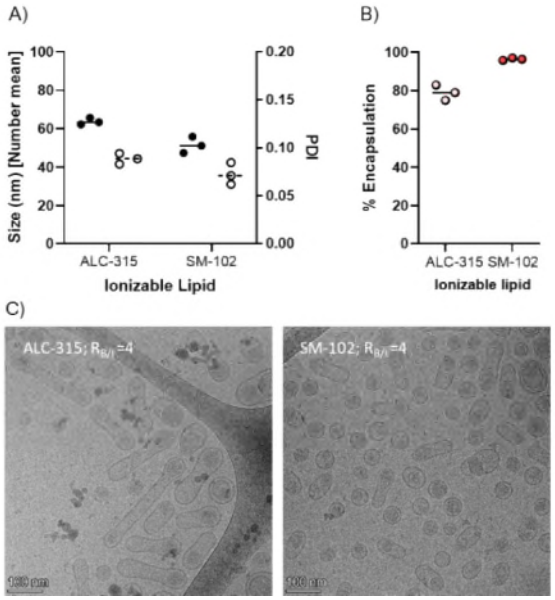


Fig S10. Liposomal LNP (ionizable lipid/ESM/cholesterol/ PEG-DMG; 20/40/40/1.5; $R_{B/I}$ = 4) formulated with ALC-0315 or SM-102 encapsulated with NanoLuc encoded mRNA at a N/P ratio of 6. A) Size in number means (nm)

determined via DLS. B) Encapsulation efficiency determined via RiboGreen assay. C) Cryo-TEM micrographs of LNP mRNA systems prepared using $R_{B/I}$= 4.

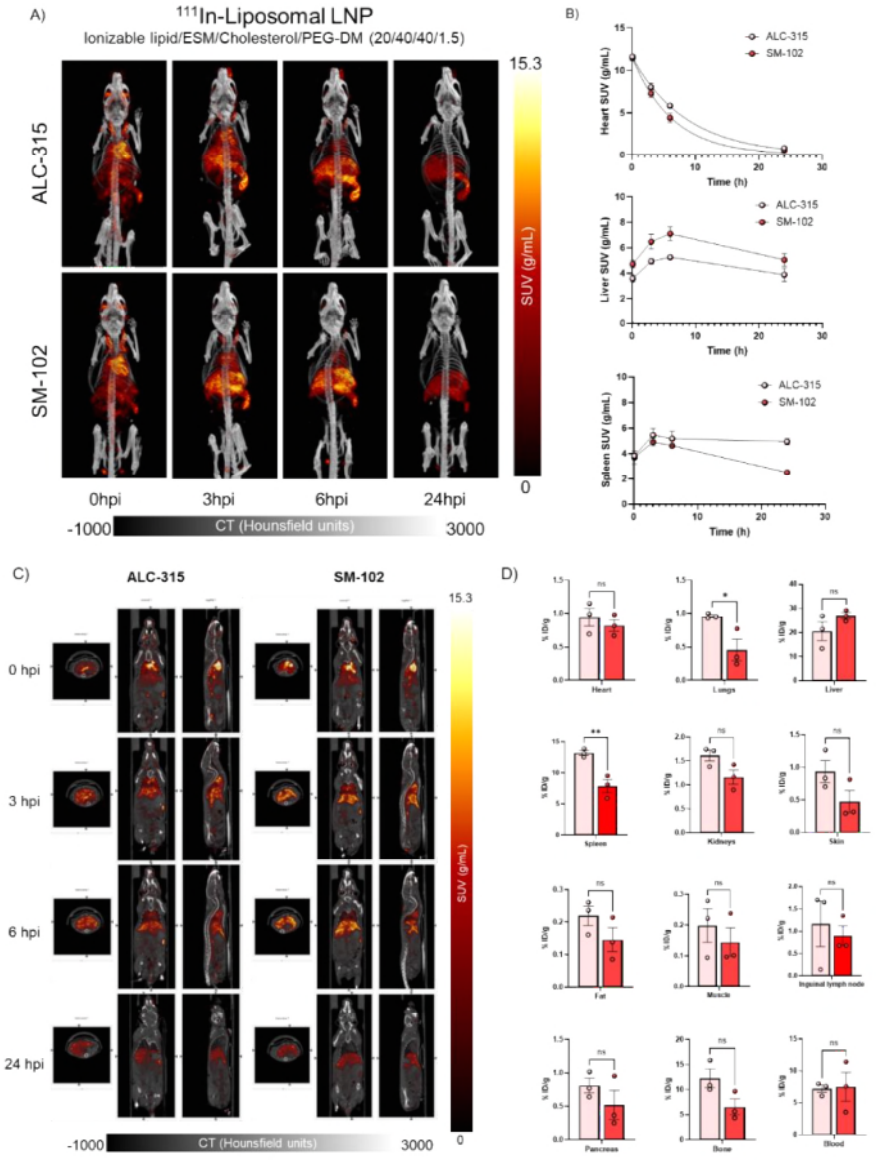


Fig S11. Radiopharmaceutical profile of ^{111}In-liposomal LNP ($R_{B/I}$: 4) formulated with either ALC-0315 or SM-102 encapsulated with NanoLuc encoded mRNA (~0.4-0.5 mCi/mouse- [^{111}In] and ~15 µg mRNA, n = 3, ±SEM) at 0, 3, 6, 24 h post-injection in CD-1 female mice. A) Representative 3D rendered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

2. The ESM-cholesterol formulation is not new. The authors' previous work has shown that extended circulation lifetimes can be achieved for liposomes composed of DSPC or sphingomyelin with

approximately equimolar levels of cholesterol. It is essential to compare these formulations (DSPC vs. ESM) to emphasize the long-circulating property afforded by the ESM-cholesterol formulation in this work.

Response: Regarding the reviewer's concerns, we have demonstrated in a recently published patent, the utility of the liposomal LNP system for mRNA using different ionizable lipids (MF019) and helper lipids (DSPC). These formulations have liposomal morphologies and enhance extrahepatic delivery.[1,2] In Chander et al' s manuscript[3], we demonstrated that the helper lipid ratio of ESM/MC3/Chol/PEG-lipid 40:33:25.5:1.5 mol/mol can increase the circulation lifetime. In the present work we used a different ratio (ESM/nor-MC3/Chol/PEG-lipid 40:20:40:1.5 mol/mol). This is a more rational ratio to use given the equimolar association of cholesterol with ESM. We have used ESM in preference to DSPC as previous studies by Webb et al[4], have directly compared liposome formulations consisting of SM/Chol vs. DSPC/Chol and show that SM/Chol systems exhibit longer circulation times. As in our present study this can be attributed to significantly lower protein adsorption (data from Webb study shown below).

Table I Summary of the *in vitro* properties of DSPC/Chol and SM/Chol liposomes

Liposome	*Hydrolysis rate*[a] *(% h^{-1})*	*Vincristine leakage*[b] *(% h^{-1})*	*Protein adsorption (μg protein mg^{-1} lipid)*
DSPC/Chol	9.11 (*0.999*)	2.7 (*0.982*)	13.7
SM/Chol	0.090 (*0.520*)	1.9 (*0.997*)	<1.75

[a]Hydrolysis at pH 2.0, 37°C (r^2 of the linear regression). [b]Leakage of vincristine incubated in plasma during 6 h at 37°C (r^2 of the linear regression).

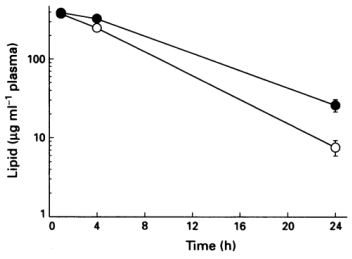


Figure 1 Amount of lipid remaining in the circulation of BDF1 mice injected with liposomes composed of DSPC/Chol (○) or SM/Chol (●). The injected dose of lipid was 20 mg kg^{-1}, corresponding to a total injection of approximately 430 μg of lipid. Data represent means (± s.e.) of three mice; where standard error bars are not visible, they are smaller than the size of the symbol.

3. Please label the organs in Figure 2B, which currently looks confusing. Also, clarify the location in mice where muscle tissues were collected.

Response: In response to the reviewer's suggestion, we have included labels of the organs in Figure 2B and clarified in the caption that the muscle tissues were collect from the left flank.

4. The 3D rendered SPECT/CT imaging used to study biodistribution (Figure 2E) does not show significant differences in extrahepatic delivery compared to Onpattro. Please revise this figure and include a statistical comparison of %ID/g in each organ.

Response: A more detailed version of the result from Figure 2E is in Figure S8 where statistical comparison has been performed. A sentence indicating where to find the statistical analysis has been included in Figure 2E's caption.

5. The authors claim that reduced plasma protein adsorption is attributed to the ESM-cholesterol liposomal formulation. To substantiate this, LNPs with other ionizable lipids (SM102, ALC0315) should be investigated in parallel to demonstrate whether the ESM-cholesterol liposomal formulation can generally reduce plasma protein adsorption.

Response: To demonstrate that the reduced protein adsorption was a consequence of the membrane properties arising from ESM/Chol, we formulated SM-102 and ALC-0315 in the liposomal formulation and examined their protein corona profile. The data showed similar profile where both ionizable lipids formulated under the $R_{B/l}$=4 with resulted in reduced protein adsorption. This new dataset has been incorporated as Fig. S15 in the SI.

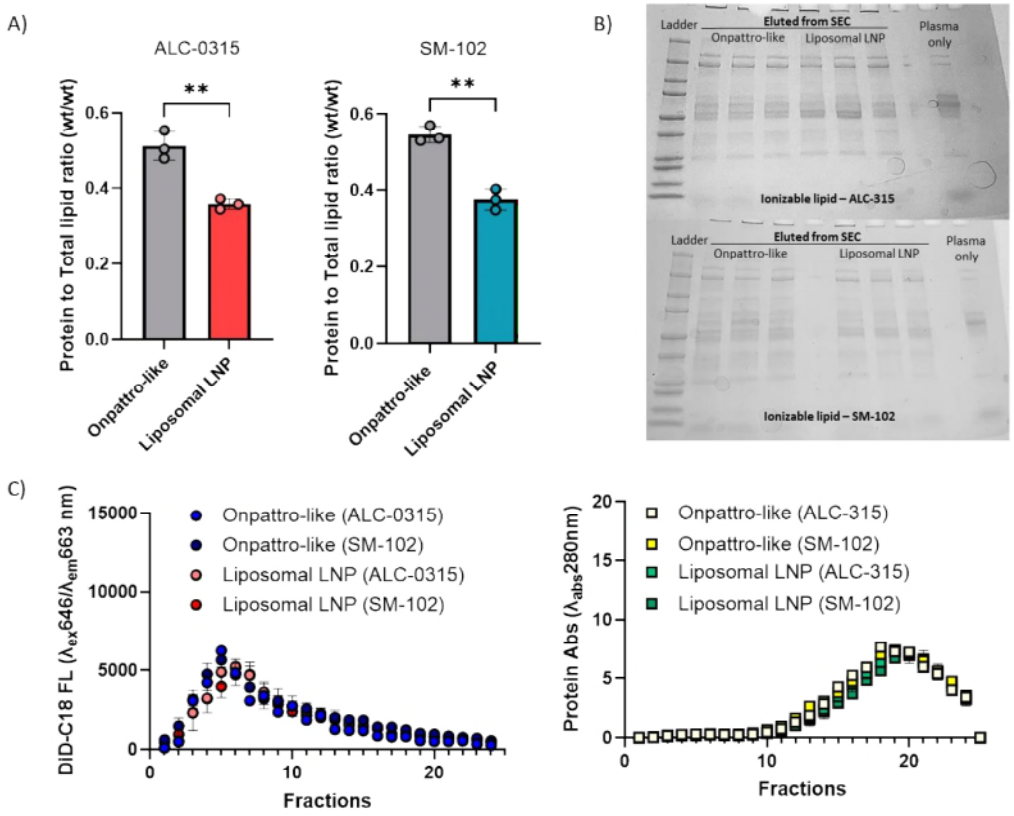


Fig. S15. Liposomal LNP (either ALC-0315 or SM-102/ ESM: cholesterol/ PEG-DMG; 20/40:40/1.5; RB/I=4) and LNP with the Onpattro composition (either ALC-0315 or SM-102/DSPC/cholesterol/DMG-PEG: 50/10/38.5/1.5) formulated with different ionizable lipids, N/P ratio of 6, were formulated, incubated with plasma 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. B) SDS–PAGE of the plasma proteins adsorbed to the surface of Onpattro 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 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). 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 0.8wt%.

6. Some helper lipids, such as DOPE, are known to increase mRNA transfection at the cost of stability. ESM may also lead to less stability compared to DSPC. A three-month stability study should be added.
Response: ESM/cholesterol mixtures give rise to highly ordered stable bilayer structures[5] and would be expected to provide a more protective bilayer structure than DOPE which adopts the non-bilayer H_{II} phase[6].To assess the long-term colloidal stability, we measured the particle size of LNP ($R_{B/I}$ of 9-1) after storage at 4°C for 63-weeks post formulation. The results show the sizes for $R_{B/I}$ 9-2.3 remain similar over this time period (<22% change in size) and the % encapsulation also remains high (>80%). In contrast, the sizes of LNP formulations with $R_{B/I\ of}$ 1.5-1 increase by 200-300% after 63 weeks of storage and encapsulation levels decrease to below 65 and 40% encapsulation respectively. While an mRNA gel analysis showed mRNA extracted from LNPs was significantly degraded after 63 weeks of storage at 4°C for all samples, the mRNA extracted from liposomal LNP ($R_{B/I=4}$) possessed the highest mRNA integrity and translatability compared to the other formulations. It may therefore be concluded

that, if anything, the presence of high levels of ESM enhances the stability of LNP mRNA systems. These new data have been included as Fig.S2 in the SI.

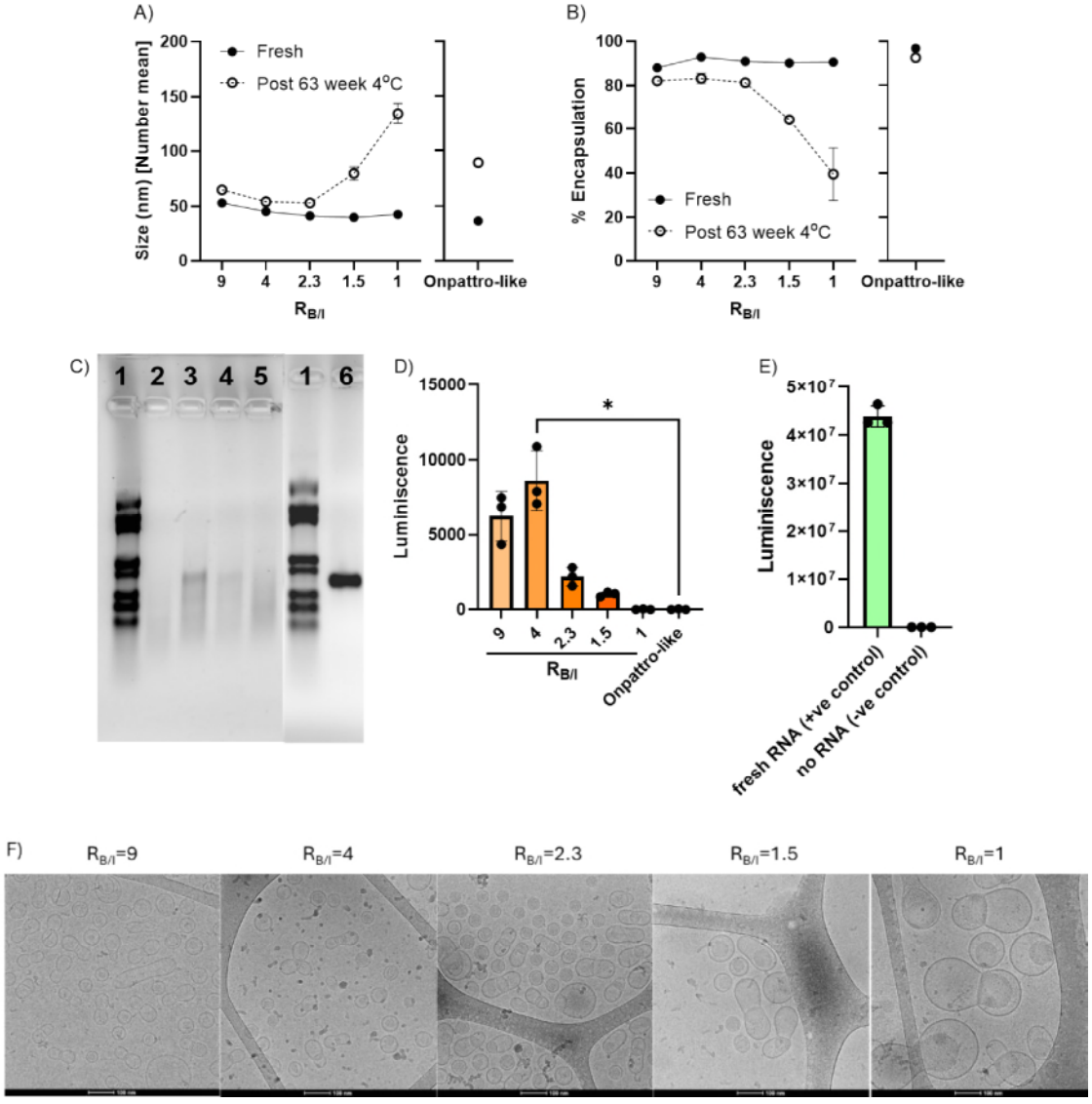


Fig S2. Stability of LNP encapsulated mRNA encoding NanoLuc after storage for 63 weeks at 4°C. LNP mRNA systems were formulated as described in Methods containing NanoLuc mRNA (808 nt; N/P=6) with a lipid composition of nor-MC3/ ESM: cholesterol/ PEG-DMG at varying ratios of bilayer lipid (ESM and equimolar 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; 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, and **p < 0.01. F) Cryo-TEM images demonstrating the change of morphologies of LNP formulated under $R_{B/I}$ of 9-1.

Minor Issues

1. In Figure S9, some green and red signals are visible in the untreated control group. Please explain this.

Response: Under the same imaging and image analysis parameters, there is a small amount of auto fluorescence observed in the green channel for the lymph node tissue, but in comparison to the treated mice the green signal is much lower.

2. In the liposomal LNP system (Figure 1A), why is the mRNA located in the aqueous phase of liposomal LNPs rather than in the ionizable solid core, as seen in typical LNPs?

Response: Previous work has shown that the ionizable lipid phase separates into a hydrophobic oil droplet at pH 7.4.[7] It is very unlikely that a negatively charged, highly hydrophilic molecule such mRNA resides in the hydrophobic environment provided by the neutral form of the ionizable lipid.

3. For the SPECT/CT reconstructed images (Figure 2D and Figure S6), it is recommended to use the same SUV range bar to facilitate the comparison of signal strengths between groups. Additionally, the positions of some organs, such as kidneys, lungs, and bones, are difficult to discern in these figures. Thus, how can the signal strength of these organs be calculated accurately based on the figures?

Response: In response to the comments, we have revised the SPECT/CT reconstructed images to show the same SUV scale bar for better comparison. The SPECT/CT contouring was done based on the CT images of the individual organ rather than through SPECT alone. Each organ was located through the sagittal, the transverse and coronal planes of the CT images. The signal strength for CT was sufficient to identify the organs with perspective ROIs. This methodology has been well established and documented for SPECT/CT imaging of nanoparticles.[8,9] To validate the SUV value, we have also cross validated with ex vivo gamma counting at 24h endpoint and have observed comparable %ID/g using the following equation (the description of the validation step has been included in Method):

$$\%\frac{ID}{g} = \frac{inj.activity \times CF}{weight.animal \times SUV} \times 100\%$$

	Ex vivo gamma counting (%ID/g)		SUV validation (%ID/g)	
	Onpattro	Liposomal LNPs	Onpattro	Liposomal LNPs
Liver	21.83±1.23	20.32±0.78	19.80±1.35	18.74±0.85
Spleen	13.57±1.00	15.10±1.17	12.89±1.82	15.61±0.10

4. There are many typographical errors in the manuscript; please revise them. For example: Line 109: “we have shown that that the proportion of LNP that are solid core can be modelled by assuming that the triolein or (neutral) ionizable lipid exists in a hydrophobic environment surrounded by a lipid monolayer with a lipid bilayer protruding from it.”

Line 471: “Fluorescnece imaging and Immunofluorescence imaging.”

Response: We have corrected these typos.

Reviewer #2 (Remarks to the Author):

Dear Editor,

Overall, the manuscript describes a clever utilization of sphingomyelin (SM) in the presence of cholesterol(Chol) within standard 4-component LNPs and how tunning the ratios between Bilayer-forming components (SM+Chol) and a MC3 analogue can improve blood circulation time and extrahepatic transfection of a surrogate NanoLuc mRNA compared to Onpattro LNP composition.

The research borrows the concept from previous long-circulating liposomes which had demonstrated some levels of extrahepatic accumulation. Further, it builds well on their group's previous paper (Kulkarni et. al., Nanoscale, 2019), where they had explored the role of helper lipid and Chol in the formation and stability of siRNA-encapsulated LNPs.

Yet, the concepts of extrahepatic delivery of mRNA via combination of helper lipid and/or ratio alterations have been explored by other researchers such as Whitehead's group (LoPresti et.al., J Control Release, 2022) or Dahlman's group (Radmand et. al., NanoLetters, 2023) in different forms.Nevertheless, similar concepts. Therefore, while the study has come up with a few LNP conditions at N:P 6 and 1.5% PEG-lipid as long circulating carriers for NanoLuc mRNA, it perhaps lacks the novelty factor to be reported in Nature Communication. My humble recommendation for this report is to perhaps be considered for more specialized Journals.
Response: The paper from the Whitehead group uses charged lipids such as DOTAP and phosphatidylserine (PS) to achieve enhanced delivery to lung and spleen. This approach is completely different from the work performed here where we are incorporating high levels of uncharged lipids (ESM) to result in liposomal structure to enhance circulation lifetimes. The presence of negatively charged lipids is well known to result in reduced circulation lifetimes for liposomes.[10] The presence of cationic lipids activates complement[11] and also results in reduced circulation lifetimes.[12] Similarly, the major result of the paper from Dahlman's group is that positively charged LNP transfect lungs more effectively. Neither paper characterized the circulation lifetimes or the morphology of their systems.

In addition, the present manuscript could benefit from some revisions as below:

1. Unfortunately, I could not locate the LNP formulation section under "Materials and Methods" on how they have formulated mRNA(808 nt) with their different lipid blends. I would recommend including detailed enough LNP formulation method.
Response: To improve clarity, we have expanded the formulation section in Materials and Methods which is also highlighted in the main text.

2. Unknowing about the formulation method, I find it interesting that Onpattro benchmark LNP with 808nt Luc mRNA has resulted in ~ 40nm (Fig 1C) particle size at N:P 6! Done several times by my group and several other credible researchers, Onpattro composition typically results in ~ 50-80 range with encapsulated siRNA or mRNA, using microfluidic micromixing followed by gentle dialysis. Given that the study reports the values as diameter, I would recommend doing n=5 independent formulations of Onpattro with and without their mRNA and report statistical analysis and PDI.
Respond: Many studies have demonstrated that when LNPs are formulated with mRNA (N:P=6) or siRNA (N:P=3), results in 40-50 nm LNP as reported by number mean from DLS analysis. This has been reported in many publications[13-20]. To address the reviewer's concern and to clarify the size

measurement, we have included more size measurement parameters (Z-average, number mean) in Table S1 and attached corresponding figures to show the distribution (intensity, volume, number and PDI). In addition to DLS data, we have included a new figure for Table S1 that summarizes the LNP size and distribution.

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 for hydrodynamic diameter in both number means and Z-average.

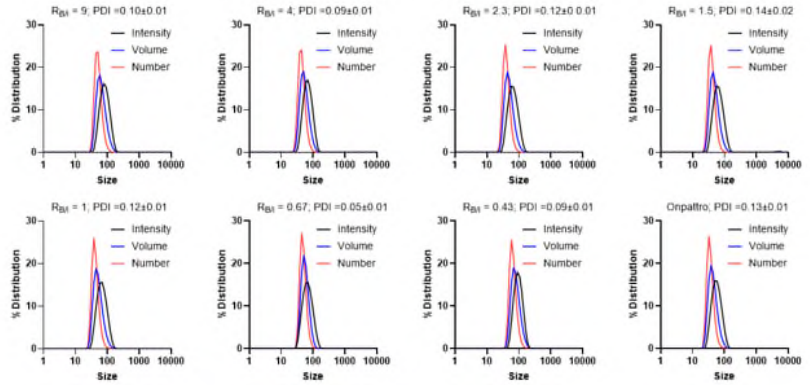
Liposomal LNPs

$R_{B/I}$	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

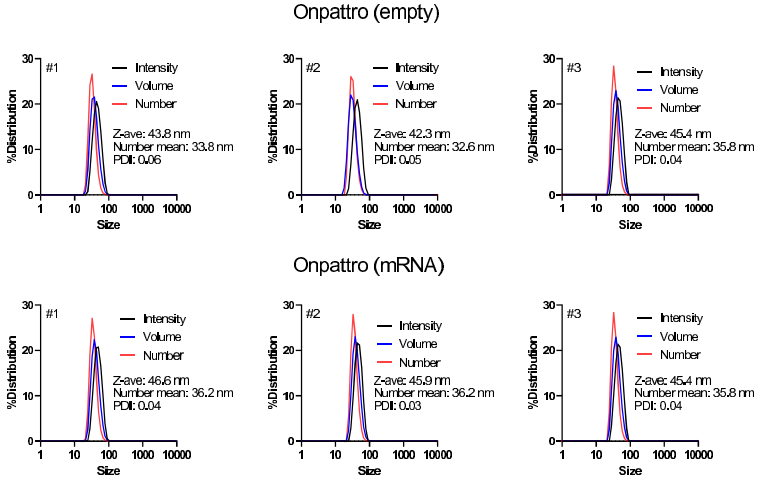
Onpattro-like

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

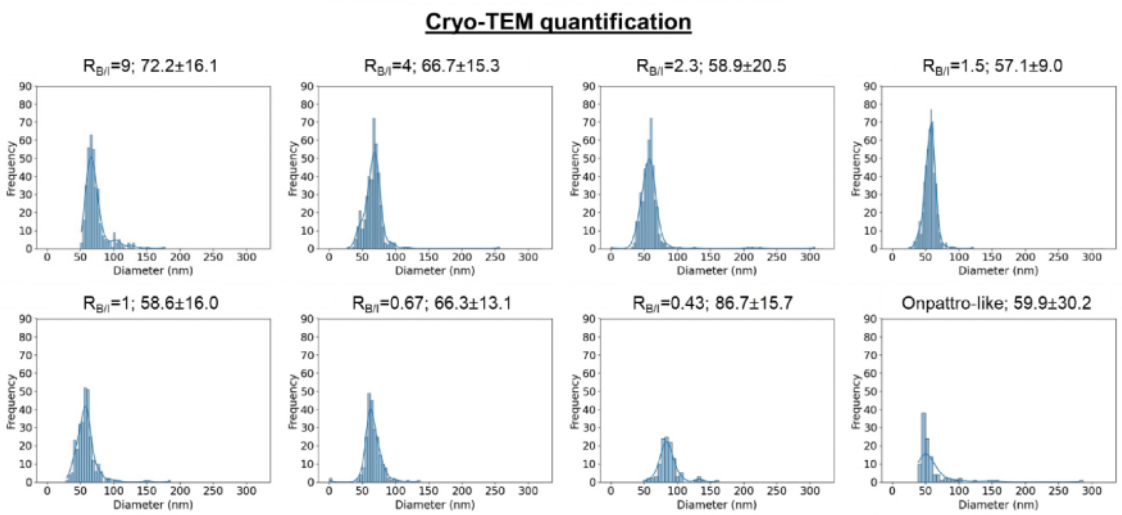


We have also provided sizing data for Onpattro composition with or without mRNA for the reviewer's reference:



3. Given the role of i-lipid molar ratio on the resulting final particle size, I wonder wouldn't the authors anticipate larger changes in ultimate size as a function drastic variations in i-lipid ratio from 10%-70%? Although qualitative and representative, yet some of the TEM images in Fig 1E do not well support the reported DLS data, e.g. R b/I = 0.43. This strengthens the skepticism on the accuracy of the reported sizes.

Respond: To address the reviewer's concern, we have now included the LNP sizes and distributions both through the number mean from the DLS data set and size measurement from the cryo-TEM images in Table S1.



4. Given that electrostatic binding of ionizable cationic lipid with mRNA backbone plays the key role in encapsulation of mRNA into the final LNP particle, how would the author explain ~85% EE for 10% i-lipid compared to 60% EE for 70% i-lipid? Also, wouldn't one expect to see change in EE as a function of change in i-lipid ratio between 10%-70%?(Fig 1B)

Response: The presence of the ionizable cationic lipid is essential to encapsulation at pH 4 where it is positively charged. The work reported here indicates that as little as 10 mol% ionizable lipid is sufficient to achieve excellent association with the small liposomes that form at pH 4.[7] In order to maintain encapsulation at pH 7.4, where the ionizable lipid is not charged and adopts a hydrophobic oil droplet core, helper lipids such as DSPC, ESM and cholesterol are required. Encapsulation levels are reduced above 60 mol% ionizable lipids, which is attributed to insufficient helper lipid to surround a hydrophilic region containing the RNA.[16]

5. Could the authors explain why N:P 6? I would recommend a systematic supplementary data on explaining why the authors have come up with 6?

Response: We did not come up with N:P 6. Many studies from many different research groups have employed N/P of 6 for LNP-mRNA[21-24], including the two clinically approved mRNA vaccines from BioNTech/Pfizer and Moderna.[25]

6. Since the suggested extrahepatic delivery approach has only been tested for Luc mRNA, would this be only specific to Luc mRNA or it could be applier for other types of mRNA regardless of translation to secreted or membrane-bound protein?

Response: Our lab demonstrated extrahepatic delivery with reporter systems (EGFP[3] and mCherry) other than Luc mRNA. However, we have yet to investigate other types of mRNA.

Reviewer #3 (Remarks to the Author):

The interesting study by Miffy et al. supports some of the previous findings from Pieter Cullis' laboratory. Namely, that increasing the proportion of the helper lipid component in LNP formulations lead to the formation of liposomal LNPs and that these LNP structures exhibit longer circulation time and higher transfection potency. In addition to provide supportive data to this LNP formulation concept, they also look into the potential mechanisms driving these attractive attributes.
Based on the mechanistic studies, including their novel warhead-model describing the interaction between the endosomal membrane and the liposomal LNPs, I believe this work could be suitable for publication in Nature Communication. However, some additions and adjustments to the manuscript, which are highlighted below are in my opinion needed before the manuscript is ready to be published.

Major comments:

1. Re. LNP composition: Previous studies by some of the authors have shown that B:I ratios of 4.2 (Kulkarni et al. ref. 17, based on DSPC and siRNA payload) and 2 (Chander et al. ref. 13, based on ESM and mRNA payload) give rise to solid core encapsulated liposome-like LNPs with increased circulation time and increased transfection potency in extrahepatic organs/tissues compared to their spherical solid LNP counterpart based on the standard B:I ratio of ~1.
Hence, the authors need to present some arguments for why it is relevant to evaluate a wide range of B:I ratios. They should also provide some rational/context as to why they decided to use ESM and nor-MC3 (contains two methylene groups less in the lipid tails than ^{the} clinical relevant MC3) instead of the standard DSPC and MC3 helper and ionizable LNP lipids. Maybe it could also be relevant to discuss the high cholesterol content in the high B:I ratio LNP formulations. Further, why are the authors not using dihydrosphingomyelin (DHSM) instead of ESM taken into account the attractive pharmacokinetics of DHSM compared to ESM as highlighted in ref.9.

Response: There are a lot of questions here. First, with regard to the range of $R_{B/I}$ ratios employed, we wanted to identify ratios at which predominant solid core within a bilayer morphology was consistently observed, settling on the $R_{B/I}$ ratio of 4. Second, the high cholesterol contents were simply chosen to match the levels of ESM, given the approximately 1:1 solubility of cholesterol in lipids such as DSPC and ESM in bilayer systems.[26-28] Third, we chose to use ESM as liposomes containing ESM exhibit extended circulation lifetimes compared to DSPC. Fourth, we chose nor-MC3 for patent reasons, the rights to MC3 are held by Arbutus. Previous work has shown that MC3 and nor-MC3 result in similar transfection levels in vivo, which can be found in this patent.[29] Fifth, we did not use dihydrosphingomyelin as it was not available to us.

2. Re. LNP biodistribution/transfection potency: The authors should discuss the biodistribution/transfection data in more detail so that the reader understand whether the increased transfection potency of the liposomal LNPs in extrahepatic tissues is driven by an overall enhanced transfection potency across organs, a organ-selective transfection potency, or is (for the most part) driven by its extrahepatic delivery capabilities when compared to the "Onpattro" formulation.
Based on a visual look at Figure 2C, it looks like the liposomal LNP-mediated transfection in the liver is more than 5x10^6, while for "Onpattro" the corresponding value is 3x10^6 RLU/g, while their corresponding 111In-radiolabeled LNP biodistribution data show similar distribution to the liver for the two different LNP formulations. These data combined suggests a higher transfection potency of liposomal LNPs compared to "Onpattro" in the liver. Maybe the ratio of the luminescence values and the

ID%/g numbers could be a useful measure in a discussion about the different transfection potency across organs/issues. As part of this discussion, I suggest the authors to discuss why the biodistribution (Figure S8) and transfection level (Figure S3) are not highly correlated.
That said, I greatly appreciate the data shown in Figure 2C and E, as I find these data more quantitative than the IVIS imaging-based data (which are associated with large error bars as shown in Fig. S3). Maybe, the authors could also discuss the differences between the data/methods presented in Figure 2C and E and the IVIS-data presented in the Supplementary Material.
Response: Correlations between biodistribution and transfection are difficult to make. Here we are mainly concerned with producing LNP mRNA systems that exhibited longer circulation lifetimes so that appreciable amounts of LNP actually reached extrahepatic tissues. As noted, a much different transfection profile was observed as a result. We believe this will be of interest to the scientific community. One logical approach would be to correlate biodistribution and expression with levels of expression of LDLR in various tissues. However, these detailed studies are really outside the scope of the present study. In passing, we should note that Figure S3 was mislabelled as an IVIS measurement but in fact reflected the luminescence resulting from ex vivo organs that were homogenized and the subsequent lysate analyzed as described in the Method section. This has been corrected in the revised manuscript.
3. Re. the biomolecular corona (BCM) studies: From the BCM data presented in Figure S11A, which include large error bars, I find it difficult to understand the significance of the stated two-fold increased biomolecular corona on “Onpattro” relative to the liposomal LNPs. How each of the single technical replicates compare (protein per ~lipid(DID) content wise) with each other would be valuable information. Further, why are the retention volume of the void fraction different in the “Onpattro” samples compared to the liposomal LNP samples? Comparing the total protein content from the pooled 3-10 fractions across the “Onpattro”, Liposomal LNP, and bare plasma (no LNP) samples could also provide some understanding of the significance of the amount of plasma proteins that are shifted toward the void volume due to their adsorption to the LNPs. Maybe such comparison is difficult due to the different void retention volumes for each of the systems? Interestingly, the data presented in Figure S11A also highlights to some extent that the liposomal LNPs are more stable (in terms of particle/DiD integrity) in plasma than the “Onpattro” formulation according to the long and rather intense DiD fluorescence tail in the chromatograms of “Onpattro” compared to the liposomal LNP formulations. Maybe that is worth highlighting?

Response: With regard to the significance of the amount of protein bound to LNP with the Onpattro formulation and the liposomal composition, experimental triplicates and a statistical analysis was performed. Each replicate is a new formulation and incubation from the gender pooled plasma samples. Samples are run in 3 different columns but with the same diameters and 20 mL of Sepharose as the stationary phase. It should also be noted that reduced levels of protein adsorption are consistent with an external bilayer composed of ESM/Chol where reduced levels of protein corona have been observed compared to DSPC/Chol.[4] We have also examined the protein adsorption for the LNP formulated at $R_{B/I}$ =4 but with other ionizable lipids (ALC-0315/SM-102), where we found similar profile see Fig.S15.

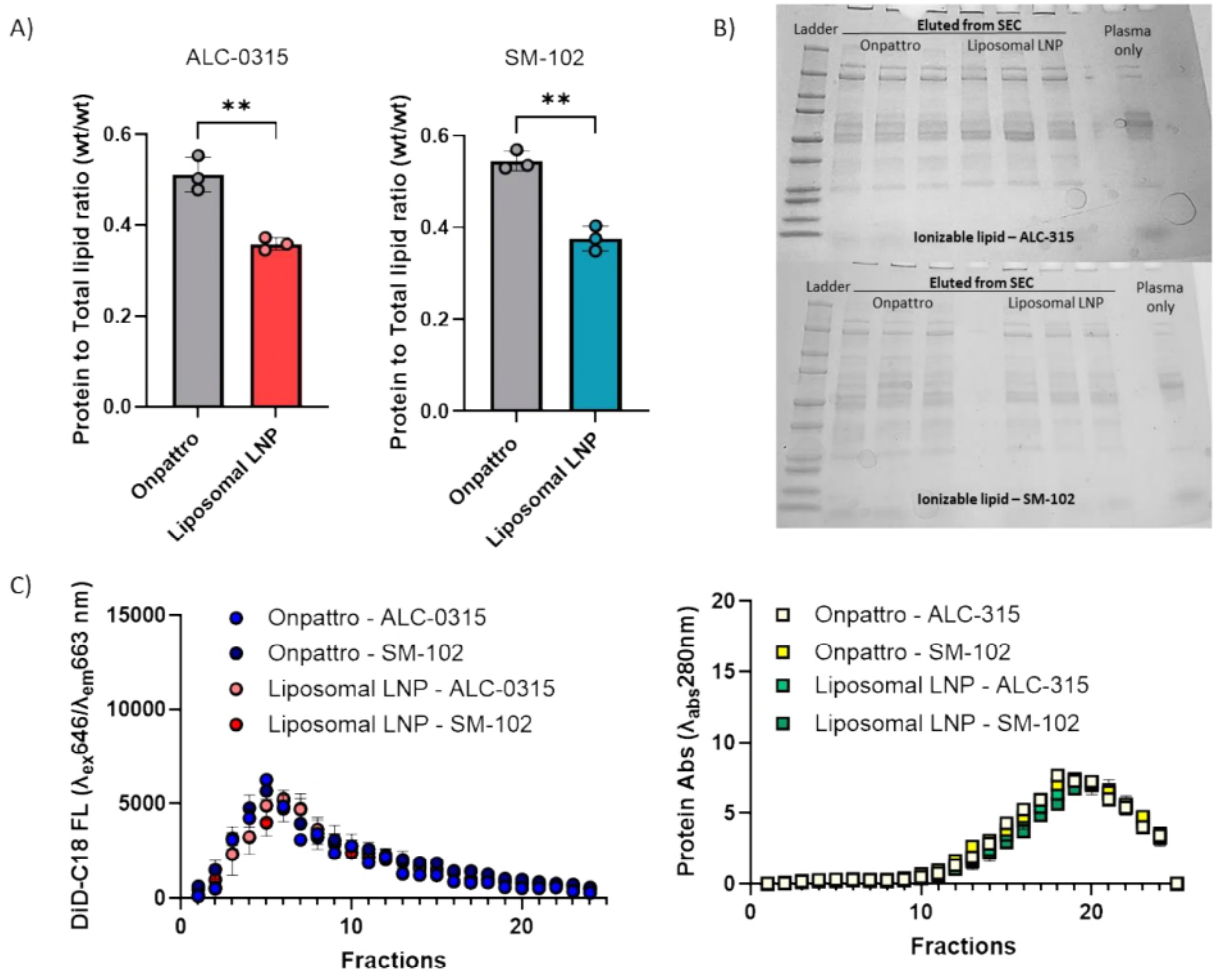


Fig S15. Liposomal LNP (either ALC-0315 or SM-102/ ESM: cholesterol/ PEG-DMG; 20/40:40/1.5; RB/I=4) and LNP with the Onpattro-like composition (either ALC-0315 or SM-102/DSPC/cholesterol/DMG-PEG: 50/10/38.5/1.5) formulated with different ionizable lipids, N/P ratio of 6, were formulated, incubated with 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. 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). 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 0.8wt%.

Moreover, we found that the replicates using this separation process were comparable, all showing higher protein adsorption with the Onpattro formulation over the Liposomal LNP formulations. This is also indicated by the statistical analysis in Figure 3A. The data showing the population abundance between each replicate is comparable between the experiments with < 3% difference per replicate, which indicate the protein population in each biological function categories was reproducible. To clarify this, we have included Table S4 in the SI.

Table S4. The proportions of proteins with distinct biological functions in the protein coronas of LNP mRNA systems with the Onpattro lipid composition as compared to the liposomal LNP 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 (%Abundance)	Liposomal LNP ($R_{B/I=4}$) (%Abundance)	Plasma Control (%Abundance)
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%

To the reviewer's comments regarding LNP stability based on the retention seems tenuous as the fractions collected vary depending on the proteins adsorbed. We have revised and included the plasma control group in Figure S11A to demonstrate this:

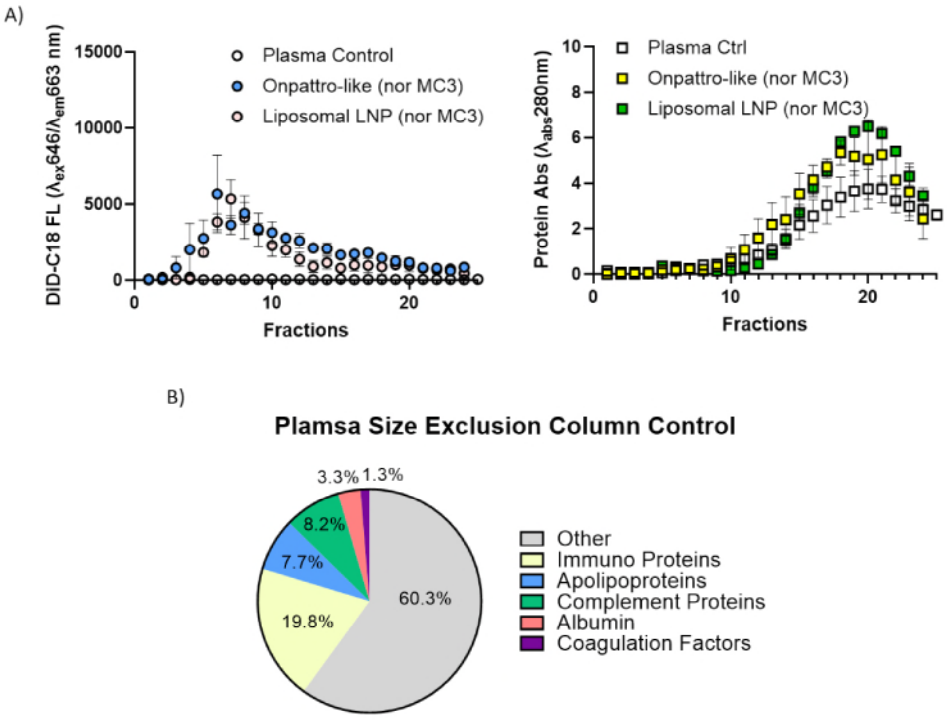


Fig S14. A) Fractions collected through size exclusion column chromatography for Plasma Control (n=4, ±SEM), Onpattro-like (MC3/DSPC/cholesterol/ PEG-DMG; 50/10/38.5/1.5) and Liposomal LNP (nor-MC3/ESM/cholesterol/ PEG-DMG; 20/40/40/1.5; $R_{B/I}$: 4) (n=3, ±SEM). Total protein measured from BCA assay and total lipid calculated from DiD-C18 absorbance measurement ($\lambda_{abs.}$ 649 nm). 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 wt%. B) The average abundance of proteins with distinct biological functions in the plasma control that has been collected after size exclusion column chromatography.

4. Re. the mechanism of endosomal escape: Regarding the "Mechanism of action for mRNA loading and intracellular delivery by liposomal LNPs" including Figure 4 A and B: Why do the authors not highlight the high degree of liposomal LNP aggregation at low pH observed in the cryo-TEM data in addition to the observed 'warheads'. I would expect that the observed LNP aggregation supports the

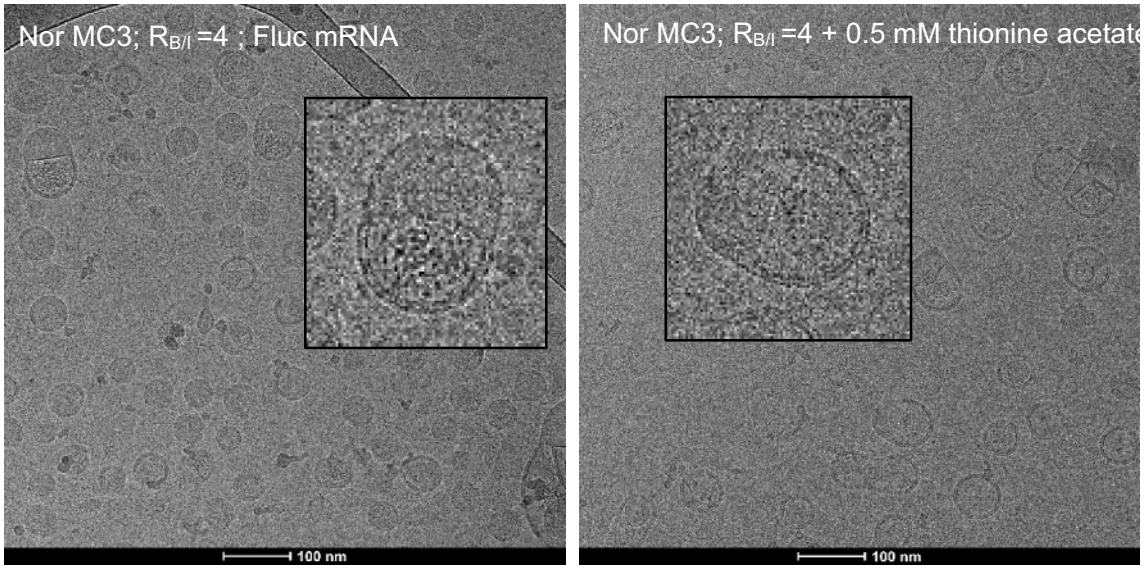
notion that liposomal LNPs are efficient in interacting with lipid membranes (in this case independent on the negatively charged endosomal lipids). Any complementary data that supports the warhead-mechanism would indeed increase the impact of this manuscript, as this part is in my mind novel.

Response: We do not feel that the aggregation observed actually reflects phenomena related to potential interactions (fusion) with the endosomal membrane as it likely reflects destabilization resulting from partitioning of the ionizable cationic lipid into the lipid bilayer as the neutral form in the oil core is converted into bilayer-forming positively charged lipid. The interaction of interest is the interaction of the positively charged LNP with the negatively charged inner membrane of the endosome. For a detailed description see Cullis and Felgner, Figure 4.[30]

5. Re. mRNA stability: In a previous study (https://onlinelibrary.wiley.com/doi/10.1002/adma.202303370) by some of the authors, they highlight that the stability of mRNA in bleb (bilayer/liposome-like) structures compared to mRNA encapsulated in spherical solid LNP structures resulted in an increased transfection potency. It would be highly relevant to discuss whether the increased transfection potency of the liposomal LNPs is also in part due to the increased stability of mRNA. I suggest the authors to evaluate where the mRNA is located (likely present in the large 'bleb'/aqueous core in the liposomal LNP structure) and the stability of the mRNA in the liposomal and spherical solid LNPs like they do in the study highlighted above. Evaluating each of the different identified positive LNP attributes would increase our understanding of which and how much each attribute contributes to (in this case) the increased transfection potency.

Response: To reviewer's comment, we have attempted to locate the mRNA with the liposomal LNP using the thionine dye staining method previous published by Brader et al.[31]

Unfortunately, we were unable to see the condensation of the mRNA within these structures. This could be due to the mRNA existing within the larger aqueous pocket with the LNP and are diffused within the LNP and are not fully visible. However, for some LNP where the phase separation adapts more like a bleb structure, we did observe some condensation effect similar to formulating the blebs with 300 mM Na-citrate.



We have previously shown that liposomal LNP can improve mRNA stability and integrity.[3] We have also included stability data after storage at 4°C for 63 weeks to show case the colloidal stability, mRNA encapsulations, mRNA stability, integrity and translatability after long term storage, see reply to Reviewer 1, point 6.

6. Re. liposome control: Would it add value to the study to evaluate a liposome formulation based on 40 ESM:40 Chol:1.5 PEG-DMG in some of the studies (e.g., the biodistribution/half-life study) to highlight the ESM-Chol-PEG-lipid surface composition and liposomal nature of the liposomal LNPs? Or would the small proportion of the ionizable lipids at the liposomal LNP surface and potential size differences make such simple comparison useless?

Response: This is an interesting idea but, as the referee mentions, is likely to be confounded by the small amount of ionizable lipid in the neutral form that is present in the lipid bilayer. The protein corona is very sensitive to the lipid composition, and it is likely that the circulation lifetime of the liposomal LNP is significantly shorter than observed for pure ESM/Chol liposomes as a result of a larger protein corona.

Minor comments:

7. Lines 23-24: Not sure whether this statement is correct, as several research groups have shown that negatively and positively charged LNPs home to the spleen and lungs and I don't believe that these types of LNP formulations exhibit a long circulation time.

Response: We have revised the wording in the abstract.

8. Lines 94-95: It is not clear what “1.5 mol ratio” (1.48) refers to in “maintaining the PEG-lipid content constant at 1.5 mol ratio”. Consider writing “1.5 molar percentage” or “1.5 mol%” instead.

Response: To keep the description of the LNP composition consistent, we referred to everything as mol ratio we prefer not to interchange the measurement units.

9. Lines 117-118: “It may be noted that the oil droplet is located either in the centre of the LNP or associated with the membrane bilayer”. Please explain why the solid core is not always associated with the LNP membrane (likely a statistical argument). Keep in mind that the cryo-TEM provides two-dimensional projection images. Hence, the solid core observed in cryo-TEM in the center of the liposomal LNPs could in principle also be associated with the LNP membrane.

Response: The reviewer is correct; the solid core is likely always associated with the surrounding bilayer either as a bulge in the bilayer or connected by a tether that is not visualized by cryo-TEM. This would account for the ready exchange between core lipid and bilayer lipid as the pH is lowered. The sentence has been reworded.

10. I am also missing a brief discussion about the data on “Onpattro” and the B:I = 1 data, as these LNP formulations are based on roughly similar lipid type ratio but differs with respect to helper (ESM vs DSPC) and ionizable (nor-MC3 vs MC3) lipid.

Response: If it is assumed that all the cholesterol in the Onpattro formulation (50/10/38.5/1.5) can participate in bilayer or monolayer formation then it has an $R_{B/I}$ of ~1. It is likely lower due to some solubility of cholesterol in the oil core, in addition the solubility of cholesterol in PC bilayers is limited above equimolar ratios.[26] There have been reports of cholesterol crystals in Onpattro-type formulations.[32] In any event it is likely that the $R_{B/I}$=1 (50/25/25/1.5) formulation is more stable than the

Onpattro formulation as there are sufficient ESM present to solubilize the cholesterol. A sentence has been added to this effect and have been highlighted in the main text.

11. The following text in the Figure 1 caption is a bit confusing: “If it is assumed that the cholesterol and DSPC reside exclusively in monolayer or bilayer environments the RB/I for this formulation is 1.03”. I assume that the B:I ratio is independent on where the lipids are localized in the LNP – it is a bulk lipid composition-based value.
Response: Note that 1.03 is wrong, should be 0.97. This $R_{B/I}$ is simply the ratio of cholesterol (38.5) and DSPC (10) divided by the amount of ionizable lipid (50) = 48.5/50 = 0.97. It assumes that cholesterol is a bilayer lipid, see caveats in point 10.

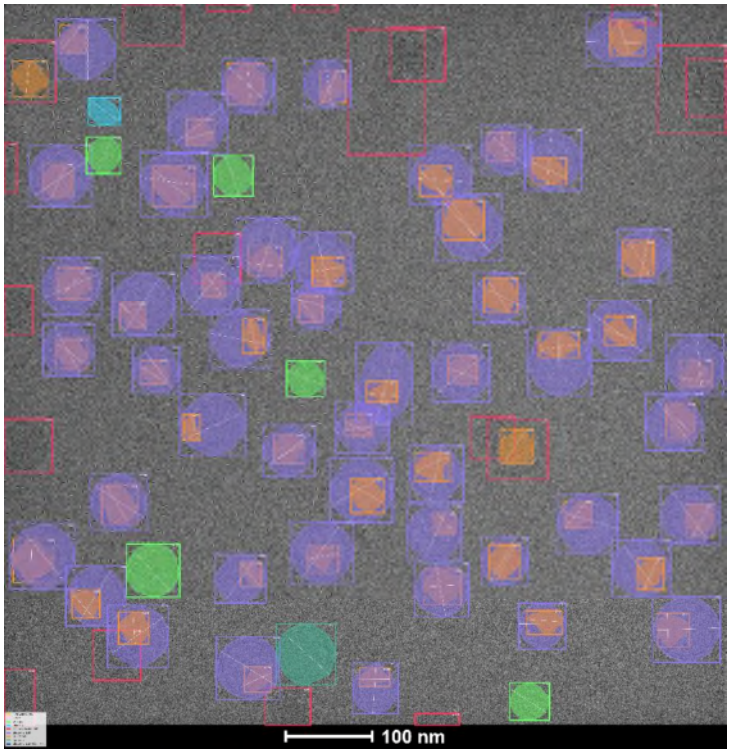
12. Figure 1D caption “D) The proportion of LNP surface area occupied by solid core, electron dense, morphology vs. the total surface area of the LNP as determined from cryo-TEM micrographs.”. I believe according to the main text “The properties of the system containing bilayer lipid to ionizable lipid ratios RB/I=4 are of particular interest as a large proportion (~84%) exhibit a bilayer structure containing a solid core component that occupies approximately 30% of the interior (Fig. 1D).” that it is the percentage of LNPs with a visible aqueous compartment that is shown in Figure 1D (not the percentage of solid core area, which is shown in Table S2). Please consider exchanging the y-label text “% Morphology” with something that is more intuitively understandable.
Please also show in the Supplementary Material how you did the segmentation (oil versus total area) using one cryo-TEM image.

Response: We apologize for the confusion as the caption was mislabelled as “surface area” when it should have been “categories of morphologies”. The Manual LNP Annotation of Cyro-TEM Images were conducted through CVAT.ai online platform (Palo Alto, CA). Each LNP was annotated for its features related to the Oil Core and liposomal LNP structure. LNPs were then annotated with a segmentation mask and categorized into either morphology within the formulation. This was classified as %Morphology indicates, we have included an equation that is highlighted in the Materials and Methods section:

$$\%Morphology = \frac{\#\ of\ LNP\ belonging\ to\ a\ class\ of\ morpholgy}{Total\ \#\ of\ LNP\ annotated}$$

Oil droplets were segmented as shown below, the % Oil droplet to LNP is calculated using the following equations to give the interior segmented cross-sectional area (CSA) of the corresponding oil droplet:

$$Proportion\ of\ oil\frac{droplet}{LNP} = \frac{CSA\ of\ oil\ droplet}{CSA\ of\ Liposomal\ LNP} + CSA\ of\ Oil\ Core\ [if\ present]$$



13. Define the “Onpattro” formulation at the beginning of the main text. Consider using the term "Onpattro-like" of ""Onpattro"" because Onpattro relies on a specific siRNA cargo and another type of PEG-lipid (PEG-C-DMG).
Response: We thank the reviewer for the suggestion, but we did define the Onpattro formulation in the first couple of sentences in the introduction. For clarity, we have revised it from Onpattro to “Onpattro-like”.

14. Line 154: Specify which lymph node(s) you analyzed.
Response: We did state in both the main text and figure caption that inguinal lymph node was analyzed.

15. Line 171-: Please provide some context to why the authors’ decided to focus on lymphoid organs when studying in vivo transfection on a cellular level.

Response: The reason why we focused on the lymphoid organs were due to our initial observation of the lymphoid organs being transfected under IVIS imaging at a marco scale level i.e. whole body imaging, which led us to then further investigate the cellular transfection through ex vivo immunohistology.

16. Adjust typos, incl:
Line 37: "(i.v,)"
Line 471: “Fluorescnece imaging …”
Fig. S9: exchange “stained with STYO13” with “stained with SYTO 13”

Fig. S11B: “Plamsa Size Exclusion …”
Response: We thank the reviewer for notifying us the typo, and they have been corrected and highlighted.

17. Table S2 is very interesting! I expect it shows that the different lipids are localized differently in an LNP. Unfortunately, I cannot figure out how the authors calculated the theoretical numbers. The authors should provide more details about the calculation.

Response: The detailed mathematical calculation spreadsheet that was part of the submission was lost during the transfer between Nature journals. We have now reintroduced it within the resubmission.

18. Re. the text “Table S1. Liposomal LNP’s lipid composition, molar ratio of bilayer lipid to ionizable lipid (RB/I), lipid molar ratio and N/P ratio.”: At low B:I ratios, the LNPs do not form liposomal LNPs. Hence, consider writing “LNPs’ lipid composition…” instead of “Liposomal LNP’s lipid composition…”.

Response: We have now revised the caption to LNP’s lipid composition.

19. Please provide some context to Fig. S2 about the different liver sizes and with and without liver in the figure caption.
Response: In response to reviewer’s comment, the liver sizes are similar as the experiment was conducted with 6-8 weeks old female mice, where their weight are within the range of 25-30g. The sizes of liver in the IVIS in situ images may be due to organ movement after the chest opening procedure and transportation to the IVIS imager.

20. You may find this new and interesting article (https://www.nature.com/articles/s41557-024-01584-z) relevant and worth discussing in your manuscript.

Response: This article is indeed an interesting study characterizing methods of constructing “concentrisomes” and their potential for exhibiting two aqueous compartments containing different active agents. We have observed similar systems.[33] However the relation of these systems to LNP developed here, which have two compartments where one is hydrophobic and the other is hydrophilic is not obvious and inclusion of such a discussion would be distracting.

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Reviewer #2 (Remarks to the Author):

With respect to the novelty factor, author argues that previous reports (exemplified by me on the initial review comments) use cationic (DOTAP) or negatively charged lipids (PS) while this study uses uncharged lipid(ESM). Plus, author borrows from previous studies to generally claim cationic lipids reduce circulation besides activating compliments.

Yet, manipulation of helper lipid type (regardless of being uncharged or charged) and ratio with cationic and/or ionizable lipids have been reported in different case scenarios. Besides authors own group (Kulkarni et. al., Nanoscale, 2019), other reports have directly reported on how Sphingomyelin (SM)-modified lipid nanoparticles improve blood circulation as the main factor for extrahepatic delivery. For instance, Webb et al (Br J Cancer, 1995, Oct:72(4): 894-904) has already shown SM/Cholestrol offer higher blood circulation than DSPC/Cholestrol liposomes when injected I.V. Another example is the most recent work by Wang et. Al. (Nat. Comm. (2024) 15:2073), where they show Cholestrol/SM lipid nanoparticles improve blood circulation.

Based on above two direct published works and my previously exemplified indirect works, I believe this research is a derivative of those studies presented as an overall approach and does not justify the novelty factor for me. Perhaps, looking at PK and PD of such nanoparticles in large animals, e.g. NHPs, would add the novelty factor.

Response: As we have previously discussed, our studies showcased that liposomal LNP formulations can yield longer circulation and extrahepatic delivery. Moreover, we show this was associated with reduced protein adsorption to the bilayer membrane structure of the liposomal LNP. Wang et al's approach is again different from the work performed here where we are incorporating high levels of uncharged lipids (ESM) without covalent conjugation while also revealing the liposomal structure by characterizing the morphological changes in LNP by titrating the $R_{B/I}$. We demonstrate such composition and structures result in the enhanced circulation lifetimes, which significantly differs from the existing literature. We further detailed the formation and release mechanism of such systems which has never been discussed in prior literature that we are aware of. Hence, we agree with other reviewers' comments regarding the novelty of this work. As to reviewer's comments regarding PK and PD with larger animals, we believe it is outside the scope of the present article, but we have acknowledge that large animal studies would add value in future studies.

In terms of authors addressing my initial review comments:

1. Authors has kindly included the Materials & Methods.

2. For this comment, author has kindly provided the detailed data. Author tends to report the particle size in both number-weighted and Z-average (cumulative intensity-weighted) distribution values. Suggested by their Z-average data, Onpattro LNP with their RNA construct shows ~ 53.9nm which corroborates with my initial review suggestion and previous reports. I strongly suggest that the author replaces Fig 1c with Z-average sizes as their provided Cryo-EM statistical data in their revision for comment 3 matches significantly closer to their Z-average. As anticipated, given the assumptions for number-weighted calculations, their mean numbers are

off in comparison to their Cryo-EM and Z-average data.

3. Author has kindly added some statistical Cry-EM data to shed light but they still have not explained why they do not observe the effect of i-lipid molar ratio.

4. Author offers a speculative argument which is a likelihood for the observed phenomenon.

5. Author has attempted single lipid/RNA ratio at N:P6, based on previous studies.

6. Author has not tried different mRNA constructs, yet study claims for all RNA delivery.

Taking all above into account, this research does not get my recommendation to be published in a Nature-family journal, rather a more specialized journal.

Response: We have addressed all of reviewer 2's initial comments by providing new data and data analysis. We have presented sizes as both a mean number and distributions and have provided sufficient scientific justification on the impact of lipid composition on encapsulation and the choice of the N/P ratio. Our studies demonstrated extrahepatic delivery with another reporter mRNA (mCherry) other than Luc mRNA. We did not claim that this is for all RNA systems but rather for mRNA systems our lab has tested.

Reviewer #4 (Remarks to the Author):

The authors present a highly interesting manuscript covering liposome-like LNPs which show improved extrahepatic delivery of mRNA. As a fusion nanomedicine system (liposome-LNP) with compartmentalisation (i.e. a LNP bleb anchored to the inner leaflet of a liposome bilayer) the research will be of considerable interest to the LNP and liposomal nanomedicine communities. The results show promise in the system with improved in vitro transfection and a broader organ biodistribution profile at the 24 h time point compared to clinically approved LNP this is argued to be linked to the improved circulation time of the liposomal-LNPs due to the ESM/Chol liposome bilayer showing lower protein adsorption with emphasis on immunoproteins.

The authors provide extensive data around a number of key aspects (e.g. CT, IVIS, protein adsorption, nanomedicine structure dynamics) and adequately address the comments of Reviewers 1 to 3. There are areas where the authors could add further detail which is both scientifically interesting, does not require further experiments and would improve the manuscript clarity.

Response: We thank reviewer 4 for these comments and have provided a point-by-point response below:

Comment 1: Reviewer 3's major comment 3 touches on the significance of the data presented

in Fig.3, particularly in the context of Fig.3A. The authors present an argument that the ESM/Chol shows reduced protein adsorption (Fig.3A and prior literature) and highlight the reduced immuno protein levels on these bilayers compared to the monolayer surface of the onpattro LNP. However, Fig.3A only presents limited significance (P-value *) with the protein heat-map data giving many broad protein groups as almost identical between the 2 systems (e.g. other, complement, albumin, coagulation factors). Equally, the authors do not discuss the differences in apolipoprotein levels between the 2 systems.

The authors should add more detail here as they have good data (1000+ proteins) that would provide further clues as to why the ESM/Chol bilayer extends circulation time. If there is an argument that part of the mechanism of hepatocyte uptake of LNPs is linked to apolipoprotein coverage then why (given the proportional increase in apolipoprotein coverage for liposomal-LNPs) do liposomal-LNPs not get cleared quickly? I can see arguments associated with increased saturation of hepatocytes allows a larger % of liposomal-LNPs to circulate in the blood, arguments associated with actual number of these proteins/liposomal-LNP etc. but the authors should discuss this briefly. Equally, given the role of complement proteins in PEG immune responses it would be interesting to explore whether these were different between the 2 systems.

Response: We further analyzed the dataset in terms of the top 10 abundant proteins and found that liposomal LNP's protein corona contains higher abundance of apolipoproteins cluster (ApoC1, ApoC4 and ApoB-100) while depleting immune response proteins (IgG and C3). The study from Liu et al. had suggested that "Orthogonal partial least squares (OPLS) multi-omics analysis revealed that acute phase, complement cascade, and coagulation function-associated proteins are less likely to be present in an apolipoprotein-abundant (and more functional) LNP corona.", similar to our observation in this study.[1] Therefore, we have now provided a brief discussion on the role of different functional proteins and how that might impact the clearance and circulation of the LNPs highlight in the MS.

Comment 2: As a technical point, the main manuscript repeatedly does not define which lymph node the authors were analysing. In the supporting information we find in Fig. S9 that it is the inguinal lymph node. However, the authors do not discuss why the choice of this lymph node and what the biodistribution data means when this lymph node is significant (e.g. 2-10% of injected dose etc.). The authors should explain their decision here.

Response: To provide better clarity, we have specified the lymph node as the inguinal lymph node in Fig 2B, captions, method and throughout the main text. A study by Pitorre et al. has demonstrated that IV injection of DiD label lipid-nanocarrier was not lymph node specific and that all the lymph nodes were targeted.[2] Therefore, we chose the inguinal lymph node over the axillary and cervical lymph node due a technical consideration as its location between the thighs and the lower abdomen is well defined for ease of dissection and extraction. The significant protein expression in the lymph node could be a result of transfected immune cells or lateral transfer of mRNA as a consequence of the liposomal LNP exhibiting longer circulation.

Comment 3: As another technical point, for many captions in the main manuscript and the supporting information it is not clear how many animals, organs, samples were used and in the

case of organs or samples whether these come from the same or different mice. As I can see from the figures, the IVIS data shows 3 mice (Fig.2A) and Fig S11 mentions that the CT images represent 3 mice. This is on the low number of mice. I would expect for most exploratory biodistribution data to be use 5-6 mice per group. The authors should acknowledge potential limitations in biodistribution data if mice numbers are low.

Response: To provide better clarity, we have further specified on the sample size in the Method section and have highlighted the addition. We acknowledge the sample size of mice of 5-6 would be more ideal. For that we have commented this limitation in the highlighted discussion section.

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