nature portfolio

Peer Review File

Microfluidics-mediated spatial control of mRNA-LNPs boosts translation and enhances vaccine potency

Corresponding Author: Dr Jiang Xu

Version 0:

Decision Letter:

Dear Dr Xu,

Thank you for submitting to *Nature Biomedical Engineering* your Presubmission Enquiry, "Reprogramming pre-formed mRNA-LNPs for self-boosting co-delivery".

As you may know, we screen Presubmission Enquiries against our editorial criteria. These editorial judgements are based on considerations of fit to the journal's scope and, when enough information is provided, of the degree of advance, broad implications, and breadth and depth of the work.

In this case, the topic of the Presubmission Enquiry is within the remit of the journal, and we would like to invite you to submit a full manuscript so that we can carry out a full editorial assessment.

I should also ask you to please fill in our reporting summary and policy checklist. (Please note that these forms are dynamic PDF files that can only be properly visualized and filled in by using Acrobat Reader.)

Both documents are aimed at ensuring good reporting standards and at easing the interpretation of results, and will be available to any reviewers. Should the manuscript be eventually published, the reporting summary will be attached to the published PDF of the paper and will also be available as supplementary information. More information is available on the editorial policies page.

Moreover, we highly recommend that you use our manuscript template. This will help you ensure that the manuscript complies with our data-presentation recommendations, that it includes all the necessary sections, and that it is structured to facilitate the assessment of peer reviewers and editors. In particular, please make sure that the manuscript provides thorough information on statistics and methods, and that the images comply with our guidelines for image integrity.

When you are ready to submit the manuscript, please upload the manuscript files as well as the reporting summary and policy checklist. DELETE_IF_COVER_LETTER_ALREADY_PROVIDED Please also upload a brief cover letter that describes the main results of the work and places it into a broader context.

Best wishes,

Filipe

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Dr Filipe Almeida
Senior Editor, *Nature Biomedical Engineering*

Version 1:

Decision Letter:

Dear Dr Xu,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "Microfluidic integrated mRNA amplification circuit (MIMAC)". The manuscript has been seen by 3 experts, whose reports you will find at the end of this message.

You will see that the reviewers appreciate aspects of the work. However, they articulate concerns about the degree of support for some of the claims and about the advance that the work represents over relevant published studies, and provide useful suggestions for improvement. We hope that with substantial further effort you can address the criticisms, increase the strength of the evidence, and convince the reviewers of the merits of the study. In particular, we would expect that a revised version of the manuscript provides:

* Compelling mechanistic evidence justifying how the mRNA located at the periphery of the nanoparticles is differentially processed versus the core mRNA after cellular uptake, as requested by Reviewer #1.

* Stronger in vivo evidence demonstrating that the hierarchical structure offers superior therapeutic outcomes, as highlighted by Reviewers #1 and #2.

* Discussion, ideally supported by evidence, on the mechanism by which their metabolic rewiring strategy enhances mRNA delivery and expression, as per comments by Reviewer #1.

* Extended in vitro evidence determining the generality of the approach, as per comments by Reviewer #1.

Shall you wish to submit a revised version of the manuscript, we would like to inform that we will likely look into recruiting an additional reviewer with an expertise in nanobiomaterials.
When you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments from all reviewers, the reporting summary, and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

*Please submit your revised manuscript in a word doc or LaTeX format.

* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).

* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

* Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.

* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We expect that you will be able to resubmit the manuscript within 25 weeks of receiving this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*.

We hope that you will find the referee reports helpful when revising the work. Please do not hesitate to contact me should you have any questions.

Best wishes,
Filipe

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Dr Filipe Almeida
Senior Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

The authors present an interesting approach to enhance mRNA-based therapeutics through the development of lipid nanoparticles (LNPs) incorporating a microfluidic integrated mRNA amplification circuit (MIMAC). This work addresses important challenges in the field of mRNA-LNP therapeutics, specifically the limitations of cellular expression efficiency and the lack of precise control over encapsulation and release kinetics.
The proposed strategy of reprogramming the intracellular environment through metabolic priming to enhance targeted mRNA translation is a notable advance in this field. The MIMAC technology demonstrates a unique approach by generating self-boosting mRNA therapeutics through metabolic modulation. The utilization of NADH dehydrogenase (ND) mRNA is particularly effective in creating a favorable metabolic environment for enhancing target mRNA expression by increasing intracellular ATP levels. This metabolic priming strategy is an interesting approach. But they are missing some key control groups to support their claims for the MIMAC technology. I have the following comments to strengthen their claims.

1. While they show the differential release kinetics of mRNA from the core vs. periphery in Figure 3g-h, this was performed PBS where mRNA-LNP is known to be stable. Can they perform this in serum-containing media?
2. How soon after the MIMAC synthesis did they perform the studies shown in Figure 3? Can you perform similar studies after storing their MIMAC at -20C vs. RT? The reason for this question is that it is not clear whether re-organization occurs between mRNA at the periphery vs. core during LNP storage.
3. Could the authors provide further clarification on how the mRNA located at the periphery versus the core of the LNP is differentially processed after cellular uptake? Specifically, the authors are asked to show whether mRNA at the periphery is more prone to rapid endosomal escape and cytosolic release, followed by translation into proteins, compared to the core-located mRNA? Please show the kinetics of reporter protein expression from peripheral mRNA vs. core-mRNA.
4. In Figure 4a-b, can the authors measure the protein levels of ND in the cells to complement the ATP level dataset? Also, the authors included a group in which Spike and ND mRNAs were delivered as a mixture of separately prepared mRNA-LNPs. However, the authors also need to test a group in which both mRNAs were co-encapsulated within the same LNP, without applying the hierarchical MIMAC strategy. This is a key control group. A comparison with this group would help clarify the specific advantage of the MIMAC approach.
5. While the authors demonstrated the advantages of the MIMAC-prepared LNPs in vitro, the in vivo validation appears limited. Although a comparison with conventional LNP was performed, further studies are needed to confirm whether the hierarchical structure can offer superior performance in vivo. It is critical to include an additional control group in all studies in Figure 5 and Figure 6, where both the metabolic primer mRNA and therapeutic mRNA are co-loaded into a single LNP without spatial control in order to better delineate the functional advantage of the MIMAC system.
6. The MIMAC strategy was primarily validated in 293T cells. Have the authors tested this system in other cell types to evaluate its generality? Especially, how might metabolic state differences across cell types influence the efficacy of MIMAC-formulated LNPs?
7. Could the authors elaborate on the mechanism by which their metabolic rewiring strategy enhances mRNA delivery and expression? For example, a recent study (J. Am. Chem. Soc. 2023, 145, 36, 19800–19811) demonstrated that co-delivery of ATP and mRNA improves protein expression by modulating intracellular energy availability and translation efficiency. How does the present study compare or differ from this approach, and can the authors run mechanistic studies further strengthen the hypothesis?
8. This study focused primarily on local (intramuscular) administration. Have the authors also evaluated the systemic delivery of their MIMAC-formulated LNP?

Reviewer #2 (Report for the authors (Required)):

The paper reports on two main features: the use of NADH dehydrogenase-encoding mRNA in LNPs and a microfluidic-based strategy for the sequential loading of this mRNA along with another mRNA encoding a therapeutically relevant protein. The authors demonstrate that microfluidic mixing prevents aggregation (or general increases in LNP size) when compared to manual mixing methods. This is an interesting observation, though not of sufficient impact to warrant publication in Nature Biomedical Engineering.
The concept of spatially differential loading of mRNAs into LNPs to achieve differential expression kinetics is also intriguing. However, this hypothesized difference in expression kinetics is insufficiently supported by data. Could this be better demonstrated, for example, by using two distinct fluorescent reporters?
The use of NADH dehydrogenase-encoding mRNA to metabolically activate cells with the aim of enhancing subsequent expression of the second mRNA is conceptually appealing. However, the in vivo advantage of the MIMAC strategy—versus simple co-encapsulation of the two mRNAs in aqueous medium followed by standard LNP formulation—is not demonstrated. These experiments are essential to determine whether the MIMAC strategy provides a real benefit over conventional co-encapsulation.
In view of these consideration I think this manuscript in not ready for publication in Nature Biomedical Engineering.

Reviewer #3 (Report for the authors (Required)):

This manuscript reports a microfluidic integrated mRNA amplification circuit (MIMAC). The authors prepared structured LNPs encapsulated with NADH dehydrogenase (ND) mRNA at the LNP periphery and therapeutic mRNA in the core via microfluidic shear forces. They designed and performed detailed experiments, and demonstrated the unique structure and

self-boosting function of the LNP. In vivo tests of cancer immunotherapy and SARS-CoV-2 prophylaxis potentiate the applications of MIMAC derived LNP. It is an original work using ND mRNA to elevate cellular ATP level and prime metabolic pathways to amplify target mRNA translation. The manuscript is well-organized. The findings and advances in this report should have broad implications in RNA delivery and therapies.The following issues should be addressed before acceptance:
1. page 4, line 6 from bottom, "Fig S7" should be Fig S6
2. page 5, line 18, "0:5:1" should be 0.5:1
3. page 6, line 8, please provide the detail to prepare "oversized Cy5-labeled LNPs (> 400 nm)" ?
4. page 7, line 1-2, do both ND/Spike and Spike/ND samples have same amount of ND or Spike?
5. SI, page 4,line 10, "The frequency f" should be "the magnitude (F) of shear force"
6. please explain why the chip was set at 75oC

Version 2:

Decision Letter:

Dear Dr. Xu,

Thank you for submitting your revised manuscript "Microfluidic integrated mRNA amplification circuit (MIMAC)" (NBME-25-0881B). Having consulted with the original reviewers (whose comment you will find at the end of this message), I am pleased to write that we shall be happy to publish the manuscript in Nature Biomedical Engineering, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements. Please do not upload the final materials and make any revisions until you receive this additional information from us.

TRANSPARENT PEER REVIEW
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Please note: we allow redactions to authors' rebuttal and reviewer comments in the interest of confidentiality. If you are concerned about the release of confidential data, please let us know specifically what information you would like to have removed. Please note that we cannot incorporate redactions for any other reasons. Reviewer names will be published in the peer review files if the reviewer signed the comments to authors, or if reviewers explicitly agree to release their name. For more information, please refer to our FAQ page.

ORCID
IMPORTANT: Non-corresponding authors do not have to link their ORCIDs but are encouraged to do so. Please note that it will not be possible to add/modify ORCIDs at proof. Thus, please let your co-authors know that if they wish to have their ORCID added to the paper they must follow the procedure described in the following link prior to acceptance: https://www.springernature.com/gp/researchers/orcid/orcid-for-nature-research

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Sincerely,

Filipe

Dr Filipe Almeida
Senior Editor, *Nature Biomedical Engineering*

Reviewer #1 (Report for the authors (Required)):

The authors have addressed the previous reviews. Overall, this is an exciting paper.
Please note that Figure 5c needs to be checked for statistical differences between the groups.

Reviewer #2 (Report for the authors (Required)):

I believe the authors have made a significant effort to address my comments and improve the quality of their manuscript. These efforts include a substantial set of new experimental data. I am pleased to recommend this paper for publication.

Reviewer #3 (Report for the authors (Required)):

The authors addressed all concerns of reviewers. Overall, MIMAC is an interesting approach to construct self-boosting mRNA delivery system with specific structure and composition and function. It may be acceptable for publication.

Version 3:

Decision Letter:

Dear Dr Xu,

I am happy to inform you that your manuscript, "Microfluidics-mediated spatial control of mRNA-LNPs boosts translation and enhances vaccine potency", has now been accepted for publication in *Nature Biomedical Engineering*.

Over the next few weeks, the figures will be checked for production quality, the text edited to ensure that it conforms to house style, and the manuscript typeset.

Our Articles are published about 40 days after the acceptance date (we recommend that you inform your institutional press office of this timeframe), and you will be notified of the actual publication date a few days in advance. Articles can be published any working day of the week, and are pushed live shortly after 10 am London time.

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To assist you in disseminating the work, as soon as the Article is published you will be able to take advantage of the Springer Nature SharedIt initiative to generate a unique shareable link to the Article that will allow anyone (with or without a subscription) to read it. Recipients of the link who are subscribers will also be able to download and print the PDF.

Thank you for having submitted this work to *Nature Biomedical Engineering*.

Best wishes,

Filipe

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Dr Filipe Almeida
Senior Editor, *Nature Biomedical Engineering*

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Rebuttal Letter

Dear Reviewers,

We are deeply appreciative of the time and expertise each of you dedicated to reviewing our manuscript. Your constructive comments have greatly helped us to improve the quality, clarity, and impact of our work. We are also grateful for the opportunity to revise our manuscript in accordance with your valuable suggestions.

We note that several key points were raised by more than one Reviewer, which we believe are central to the advancement and validation of our MIMAC platform. In particular:

1. The necessity of comparing MIMAC with conventional co-delivery strategies (e.g., Co-encapsulation and Co-transfection) was emphasized by multiple Reviewers. In response, we have performed comprehensive in vitro and in vivo comparisons, which clearly demonstrate the superior performance of MIMAC in enhancing metabolic priming, target protein expression, and therapeutic efficacy.

2. The validation of differential mRNA expression kinetics using distinct fluorescent reporters was another common suggestion. We have now included dual-luciferase (Firefly and Renilla) tracking experiments, which confirm the staggered expression profiles enabled by the layered architecture of MIMAC mRNA-LNPs.

3. Enhanced in vivo validation with additional control groups and administration routes were recommended. We have expanded our tumor and SARS-CoV-2 vaccine studies to include Co-encapsulation and Co-transfection groups, and evaluated systemic delivery, thereby providing a more rigorous assessment of MIMAC's translational potential.

We have point-to-point replied to all your comments through extensive additional experiments, detailed mechanistic studies, and clarifications throughout the revised Manuscript and Supplementary Information. We believe that these revisions have significantly strengthened our work and more clearly highlight the novelty and broad applicability of the MIMAC platform.

Thank you once again for your time and guidance.

Yours sincerely,

Jiang Xu, Ph.D.

On behalf of all authors

Reviewer #1 (Report for the authors (Required)):

The authors present an interesting approach to enhance mRNA-based therapeutics through the development of lipid nanoparticles (LNPs) incorporating a microfluidic integrated mRNA amplification circuit (MIMAC). This work addresses important challenges in the field of mRNA-LNP therapeutics, specifically the limitations of cellular expression efficiency and the lack of precise control over encapsulation and release kinetics.
The proposed strategy of reprogramming the intracellular environment through metabolic priming to enhance targeted mRNA translation is a notable advance in this field. The MIMAC technology demonstrates a unique approach by generating self-boosting mRNA therapeutics through metabolic modulation. The utilization of NADH dehydrogenase (ND) mRNA is particularly effective in creating a favorable metabolic environment for enhancing target mRNA expression by increasing intracellular ATP levels. This metabolic priming strategy is an interesting approach. But they are missing some key control groups to support their claims for the MIMAC technology. I have the following comments to strengthen their claims.

Reply: We are profoundly grateful to your thorough and insightful evaluation of our manuscript, as well as for these exceptionally constructive suggestions. Your comments have not only highlighted the strengths and novelty of our MIMAC platform but have also clearly guided us in strengthening the experimental rigor and translational impact of our study. Your deep understanding of mRNA therapy and metabolic engineering has greatly helped us in clarifying key mechanistic aspects and expanding the scope of our validations.

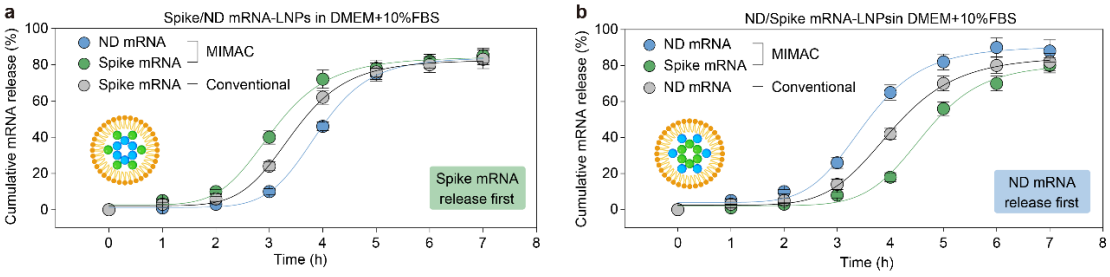
In response to your recommendations and concerns, we have performed comprehensive additional experiments, including serum-based release profiling, stability assessments under different storage conditions, detailed kinetic analyses of peripheral vs. core mRNA expression, NADH dehydrogenase activity assays, critical Co-encapsulation and Co-transfection controls, in vivo comparative studies across multiple vaccine models, cross-cell-line validation, mechanistic comparisons with exogenous ATP delivery, and preliminary systemic delivery evaluation. These additions have substantially bolstered our claims and unequivocally demonstrated the unique advantages of the MIMAC system in achieving spatiotemporally controlled mRNA delivery and enhanced therapeutic efficacy.

1. While they show the differential release kinetics of mRNA from the core vs. periphery in Figure 3g-h, this was performed PBS where mRNA-LNP is known to be stable. Can they perform this in serum-containing media?

Answer: Thank you for pointing out the difference between PBS and serum media. We have now conducted additional experiments to further investigate the release kinetics of mRNA from LNPs in a serum-like condition (DMEM+10% FBS, **revised Supplementary Fig. S15**).

Collectively, the differential release kinetics of mRNA from core versus peripheral regions of MIMAC mRNA-LNPs in serum-like media maintained analogous relative trends to those observed in PBS, though with significantly accelerated release rates. For MIMAC Spike/ND mRNA-LNPs (**revised Fig. S15a**), peripheral Spike mRNA demonstrated rapid release (~40% cumulative release at 3h) while core-localized ND mRNA exhibited delayed release (~10% at 3h), contrasting with conventional Spike mRNA-LNPs showing intermediate release (~24%). Similarly, in MIMAC ND/Spike mRNA-LNPs, peripheral ND mRNA achieved ~26% release at 3h whereas core Spike mRNA released only ~8%, compared to ~14% release in conventional controls (**revised Fig. S15b**). These new serum-based data provided robust experimental proof of distinct release kinetics of core and peripheral mRNAs within MIMAC architectures, which was consistent with our original PBS-based characterizations.

We have made corresponding modifications in the revised Manuscript (**Pages 6&7**, tracking mode) and added experimental protocols in the revised Supplementary Information (**Page 9&27**, tracking mode).



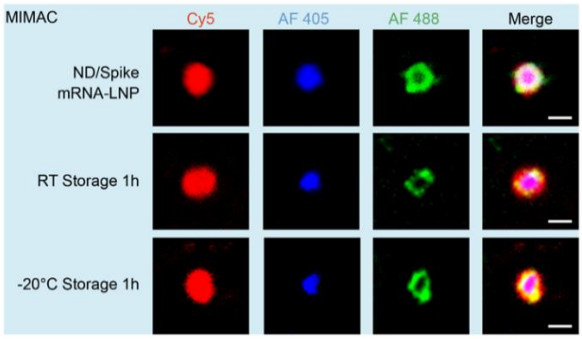
Revised Figure S15. Accelerated in vitro release profiles of MIMAC LNPs in serum-like media (DMEM+10% FBS): (a) Spike/ND mRNA-LNPs displaying rapid Spike mRNA release (1-5 h) followed by delayed ND mRNA release (3-6 h) and (b) Reverse sequential release in ND/Spike mRNA-LNPs (ND mRNA: 2-6 h, Spike mRNA: 3-7 h).

2. How soon after the MIMAC synthesis did they perform the studies shown in Figure 3? Can you perform similar studies after storing their MIMAC at -20C vs. RT? The reason for this question is that it is not clear whether re-organization occurs between mRNA at the periphery vs. core during LNP storage.

Answer: We sincerely appreciate your attention to this experimental detail. In our original study, all confocal imaging and in vitro experiments shown in Fig. 3 were conducted immediately after MIMAC synthesis (within 5 minutes post formulation). Following your suggestion, we prepared two fresh batches of MIMAC ND/Spike mRNA-LNPs and stored them at -20°C and room temperature (RT) for 1 hour, respectively before analysis. Confocal imaging revealed no

structural differences between freshly synthesized MIMAC mRNA-LNPs and those stored under either condition—all exhibited the same distinct layered architecture (**revised Fig. S14**). This confirms that the hierarchical organization of MIMAC mRNA-LNPs does not occur in storage and also demonstrates that the observed structural fidelity is stable under short-term storage conditions.

We hope these new experiments can adequately address your question. Revisions have been incorporated into the revised Manuscript (**Pages 6&22**, tracking mode) and Supplementary Information (**Page 26**, tracking mode).



Revised Figure S14. Short-term storage stability of MIMAC LNPs. Fresh MIMAC ND/Spike mRNA-LNPs maintain similar structured constructures of dual mRNAs after being stored at room

3. Could the authors provide further clarification on how the mRNA located at the periphery versus the core of the LNP is differentially processed after cellular uptake? Specifically, the authors are asked to show whether mRNA at the periphery is more prone to rapid endosomal escape and cytosolic release, followed by translation into proteins, compared to the core-located mRNA? Please show the kinetics of reporter protein expression from peripheral mRNA vs. core-mRNA.

Answer: We sincerely appreciate this insightful question regarding expression kinetics of multiple mRNAs in MIMAC LNPs, which was also raised by other Reviewers. Following your suggestions, we have prepared a dual-luciferase tracking system of Firefly (F) and Renilla (R) mRNA luciferases in two different MIMAC mRNA-LNP architectures: F/R-LNPs (peripheral Firefly mRNA) and R/F-LNPs (peripheral Renilla mRNA).

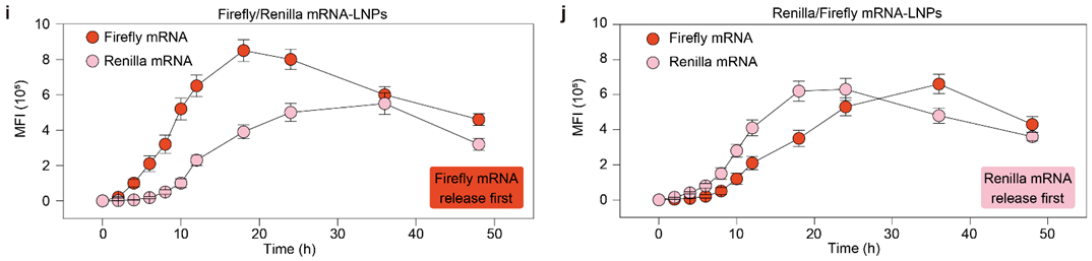
Briefly, in vitro transfection of 293T cells demonstrated (**revised Fig. 3i-j**):

(1) In F/R-LNPs, peripheral Firefly mRNA exhibited significantly accelerated expression kinetics (detectable at 2 h vs. 6 h for core-localized Renilla mRNA), reaching peak expression at 18 h (vs. 36 h for core Renilla mRNA).

(2) In R/F-LNPs, peripheral Renilla mRNA showed comparably rapid translation initiation (2

h vs. 6 h for core-localized Firefly mRNA) with plateau attainment at 18-24 h (vs. 36 h for core Firefly mRNA).

These results establish that surface-localized mRNA undergoes preferential endosomal escape upon cellular uptake, thereby enabling expedited cytosolic access and translation. The staggered expression kinetics confirms MIMAC's unique capability for temporally programmable payload release. We have updated figure and textual contents in the revised Manuscript (**Pages 7**, **21&22**, tracking mode) and added related protocols to the revised Supplementary Information (**Page 10**, tracking mode).



Revised Figure 3. In vitro expression kinetics of **i**, MIMAC F/R-LNPs (peripheral Firefly mRNA/core Renilla mRNA) and **j**, MIMAC R/F-LNPs (peripheral Renilla mRNA/core Firefly mRNA). Peripheral mRNA consistently demonstrates accelerated translation, with detectable expression by 2 h (vs. 6 h for core mRNA) and peak levels at 18–24 h (vs. 36 h for core mRNA).

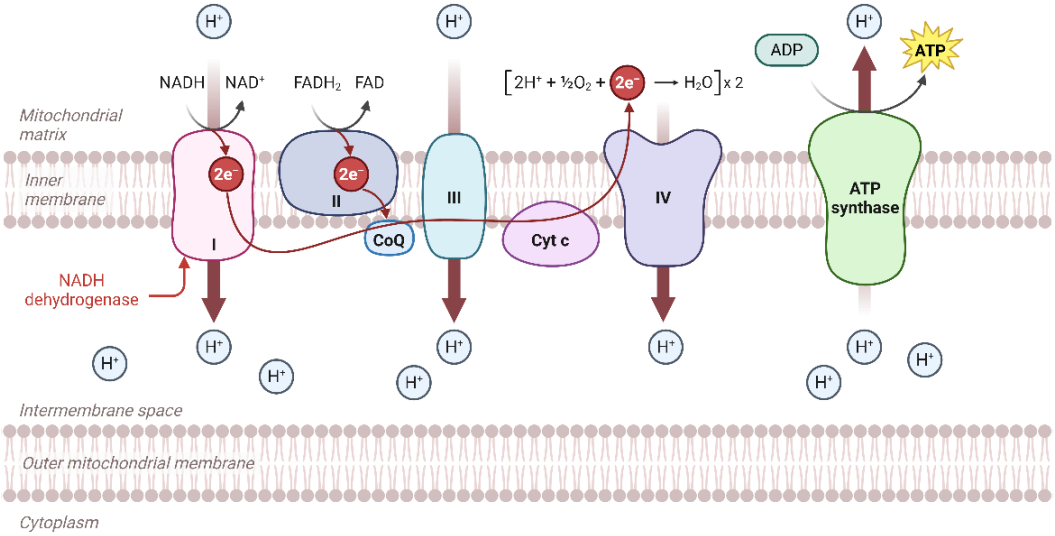
4. In Figure 4a-b, can the authors measure the protein levels of ND in the cells to complement the ATP level dataset?

Answer: We fully agree with you that quantification of NADH dehydrogenase (ND) is important to complement our ATP data. Following your recommendation, we conducted additional experiments using a commercial assay kit (AIDISHENG Biotech, Cat#ADS-F-FM006) and defined 1 NADH dehydrogenase enzyme activity unit = 1 nmol NADH consumed per minute per 10^4 cells).

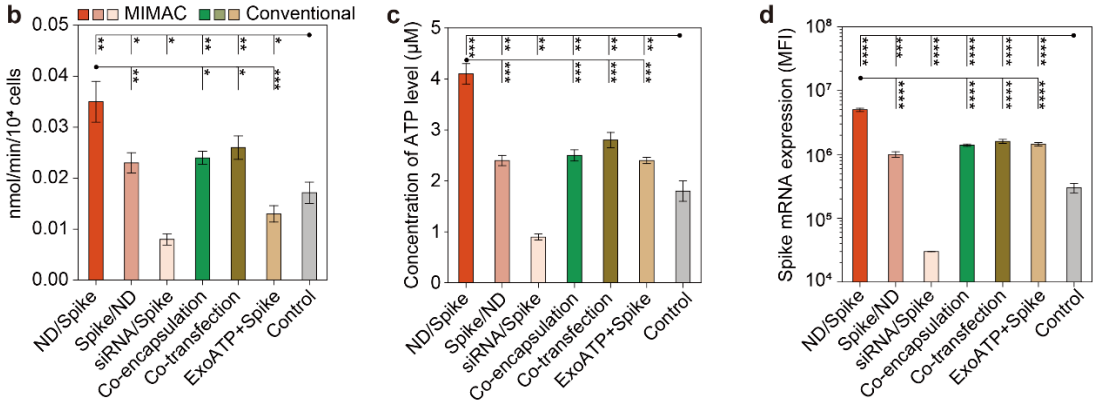
Our new findings include: (1) control 293T cells treated with only Spike LNPs exhibited baseline activity of 0.017 U; (2) MIMAC ND/Spike LNPs demonstrated the highest activity (0.035 U, $p<0.01$ vs. control), while MIMAC siRNA/Spike LNPs showed minimal activity (0.008 U, $p<0.05$ vs. control); and (3) MIMAC Spike/ND, Co-transfection, and Co-encapsulation exhibited intermediate activity (0.023 to 0.026 U, $p<0.05$ vs. MIMAC ND/Spike). Interestingly, the exogenous ATP delivery group (ExoATP + Spike) displayed reduced enzyme activity (0.013 U, $p<0.05$ vs. control). We attribute this observation to a fundamental mechanistic distinction between the two strategies: exogenous ATP delivery directly elevated intracellular ATP levels, which exerted feedback inhibition to reduce the flux through upstream metabolic pathways, thereby suppressing the activity of ND (**revised Fig. S1**). In contrast, the MIMAC strategy enhanced ATP production by directly increasing NADH dehydrogenase levels,

thereby increasing enzyme activity through metabolic pathway activation.

These results highlight two critical features of the MIMAC platform: (1) the presence of ND mRNA directly correlates with enhanced ND activity and ATP production, and (2) the sequence of mRNA release critically influences both enzyme activity and downstream protein expression. Mechanistically, the MIMAC strategy sustains enzymatic pathways by increasing ND levels, whereas exogenous ATP delivery bypasses these pathways, leading to suppressed enzyme activity despite elevated ATP levels. These latest results are presented in **revised Fig. 4b-d** and Manuscript (**Pages 7, 8, 23&24**, tracking mode), with detailed protocols updated in revised Supplementary Information (**Pages 8&13**, tracking mode).



Revised Figure S1. The role of NADH dehydrogenase (ND) in ATP synthesis. ND catalyzes NADH to NAD^+ and pumps H^+ for ATP generation. The illustration is designed via Biorender.



Revised Figure 4. b, ND enzymatic activity, **c**, Cellular ATP levels, and **d**, Spike protein expression at 24 h post-transfection of MIMAC and conventional mRNA-LNPs.

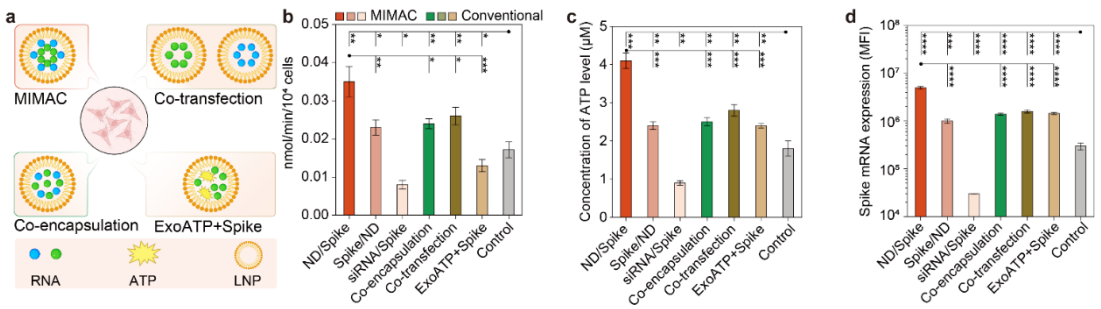
Also, the authors included a group in which Spike and ND mRNAs were delivered as a mixture of separately prepared mRNA-LNPs. However, the authors also need

to test a group in which both mRNAs were co-encapsulated within the same LNP, without applying the hierarchical MIMAC strategy. This is a key control group. A comparison with this group would help clarify the specific advantage of the MIMAC approach.

Answer: We are truly grateful that you highlighted this crucial gap in our initial experimental design. In the revised Manuscript, in addition to the Co-encapsulation group you recommended, we have also included experimental data from the exogenous ATP delivery group, as suggested in Q7 (which will be discussed in detail in our response to that comment). As a result, the study now comprises six experimental groups and one control group, which is now illustrated in the **revised Fig. 4a**.

The comparison between the Co-encapsulation and MIMAC strategies offers direct evidence supporting the superiority of our system. Firstly, the two strategies differ in their ability to enhance cellular ND levels: the Co-encapsulation group reached 0.024 U, whereas the MIMAC ND/Spike LNPs group achieved 0.035 U, compared to the control value of 0.017 U. Importantly, the difference between the Co-encapsulation and MIMAC groups was statistically significant ($P<0.05$, **revised Fig. 4b**). Secondly, whereas Co-encapsulation of ND and Spike mRNAs only moderately elevated intracellular ATP levels (1.4-fold increase; $p<0.01$), MIMAC LNPs achieved a significantly higher 2.3-fold enhancement ($p<0.001$, **revised Fig. 4c**). This stark contrast in cellular metabolic states ultimately led to profoundly different expression profiles of the target mRNA: while Co-encapsulation improved Spike protein expression by 4.7-fold, MIMAC LNPs exhibited a dramatically greater 16.7-fold increase relative to controls ($p<0.0001$ between Co-encapsulation and MIMAC, **revised Fig. 4d**).

These findings underscore the unique advantages of the MIMAC strategy, which enables more effective enhancement of intracellular ND activity, ATP level, and subsequent protein expression than conventional Co-encapsulation delivery. We appreciate your constructive suggestion, which has allowed us to rigorously validate translational potential of our platform for mRNA-based therapies. Related modifications have been incorporated in the revised Manuscript (**Pages 7, 8, 23&24**, tracking mode) and Supplementary Information (**revised Figs. S16-S18**, **Pages 4, 28-30**, tracking mode).



Revised Figure 4. a, Schematics of MIMAC and conventional co-delivery strategies. MIMAC formulations include ND/Spike (ND mRNA releasing first to boost ND), Spike/ND (Spike mRNA releasing first), and siRNA/Spike (siRNA suppressing ND releasing first); conventional groups consist of Co-transfection (dual mRNAs in separate LNPs), Co-encapsulation (dual mRNAs randomly in same LNPs), and ExoATP (Spike LNPs with exogenous ATP supplement), with Spike mRNA-LNPs alone as the control. **b**, ND enzymatic activity, **c**, Cellular ATP levels, and **d**, Spike protein expression at 24 h post-transfection of MIMAC and conventional LNPs.

5. While the authors demonstrated the advantages of the MIMAC-prepared LNPs in vitro, the in vivo validation appears limited. Although a comparison with conventional LNP was performed, further studies are needed to confirm whether the hierarchical structure can offer superior performance in vivo. It is critical to include an additional control group in all studies in Figure 5 and Figure 6, where both the metabolic primer mRNA and therapeutic mRNA are co-loaded into a single LNP without spatial control in order to better delineate the functional advantage of the MIMAC system.

Answer: We are grateful for your valuable insights concerning the in vivo validation of the MIMAC system. As recommended, we have added two critical groups to all in vivo experiments: (1) Co-encapsulation (ND and therapeutic mRNAs randomly encapsulated in the same LNPs) and (2) Co-transfection (mixture of separately prepared ND mRNA-LNPs and therapeutic mRNA-LNPs). The results across both tumor (**revised Fig. 5**) and SARS-CoV-2 vaccine (**revised Fig. 6**) models demonstrate the superior performance of the MIMAC strategy compared to conventional co-delivery approaches.

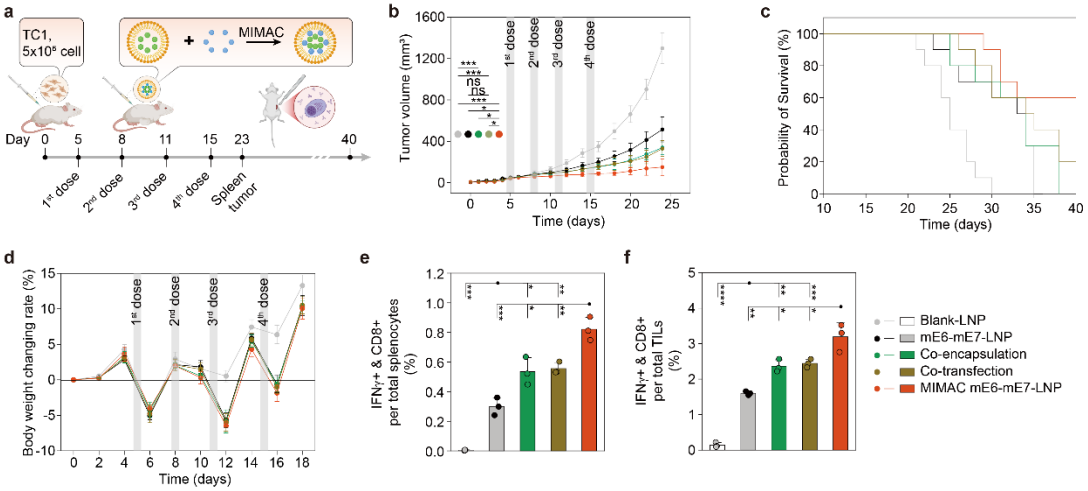
In the tumor model, the Co-encapsulation and Co-transfection groups showed intermediately enhanced efficacy compared to the conventional LNP that only contained therapeutic mE7-mE6 mRNA: Co-encapsulation achieved 74% tumor growth inhibition by Day 24 (vs. 60% in controls), while Co-transfection reached 75% inhibition. However, both groups underperformed relative to MIMAC's 90% inhibition (**revised Fig. 5b**). Survival analysis further highlighted this advantage: Co-encapsulation and Co-transfection groups retained 0% and 20% survival on Day 38 and Day 40, respectively, compared to 60% survival with MIMAC (vs. 0% in controls on Day 35, **revised Fig. 5c**). Regarding safety profiles, both Co-encapsulation and Co-transfection groups exhibited only transient physiological perturbations, with body weight changes showing no statistically significant differences ($P>0.05$) compared to conventional mE6-mE7-LNP and MIMAC formulations (**revised Fig. 5d**). In cellular immunity assessments, mice treated with Co-encapsulation and Co-transfection formulations exhibited $IFN\gamma^{+}CD8^{+}$ T-cell frequencies of 0.54% and 0.56% in splenocytes (vs. 0.30% for conventional group) and 2.37% and 2.45% in tumor-infiltrating lymphocytes (TILs) (vs. 1.60%), respectively. These values proved statistically lower than the 0.81% (splenocytes) and

3.2% (TILs) achieved by MIMAC-treated cohorts (**revised Fig. 5e-f**).

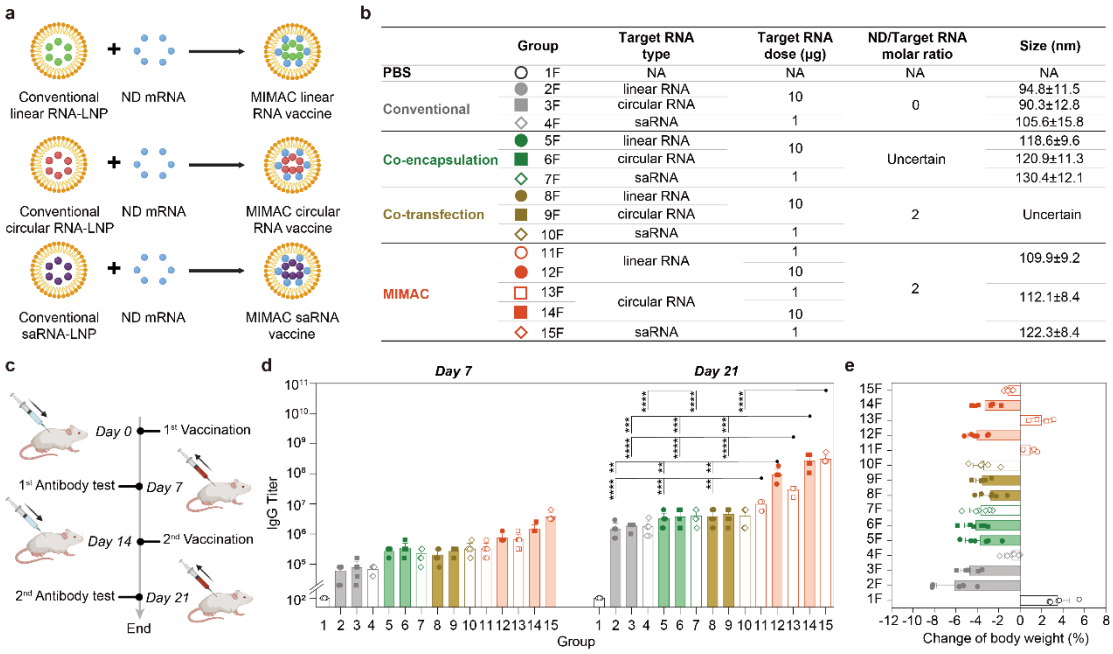
For the SARS-CoV-2 vaccine, we have now incorporated six additional cohorts: Co-encapsulation (5F-linearRNA, 6F-circRNA, 7F-saRNA) and Co-transfection (8F-linearRNA, 9F-circRNA, 10F-saRNA) with ND mRNA, alongside conventional controls containing only therapeutic RNAs (2F–4F) and MIMAC (12F–15F, **revised Fig. 6b**). Across all RNA formats (linear, circular, self-amplifying), Co-encapsulation and Co-transfection outperformed single-mRNA controls but remained inferior to MIMAC. For example, in IgG antibody titers (GMT): Co-encapsulation (5F) showed 3.20×10^6 and Co-transfection (8F) showed 3.73×10^6, respectively, whereas MIMAC exhibited 9.17×10^7 (12F, **revised Fig. 6d**). Similar trends were observed for circular and self-amplifying RNAs, with MIMAC achieving 2.70×10^8 and 3.06×10^8 GMT versus 5.46×10^7 and 6.12×10^7 GMT for Co-encapsulation/Co-transfection. These results confirm the superiority of MIMAC's spatial and temporal control over conventional mRNA co-delivery.

Collectively, the concordant findings from in vivo tumor therapy and SARS-CoV-2 vaccine studies confirm MIMAC's advantages over conventional co-delivery strategies, consistent with our in vitro results as you suggested in Q4. We attribute the advantages of MIMAC to three key factors: (1) ND mRNA enhances metabolic priming, which boosts therapeutic mRNA expression even in Co-encapsulation and Co-transfection groups compared to conventional single-mRNA controls; (2) Sequential release of ND mRNA first ensures optimal metabolic conditions before therapeutic payload delivery; (3) Although these methods have little difference in their impacts on cell viability (**revised Fig. S16**), MIMAC guarantees both mRNA co-packaging within same LNPs (superior to stochastic Co-encapsulation, **revised Fig. S17**) and synchronized delivery to same cells (addressing Co-transfection limitations, **revised Fig. S17**).

We reiterate profound gratitude for your expert guidance on the in vivo experiments, which have substantially enhanced our study's rigor and credibility. Corresponding revisions have been systematically incorporated throughout the revised Manuscript (**Pages 10-12&25-28**, tracking mode) and Supplementary Information (**Pages 4, 10, 11, 28&29**, tracking mode).

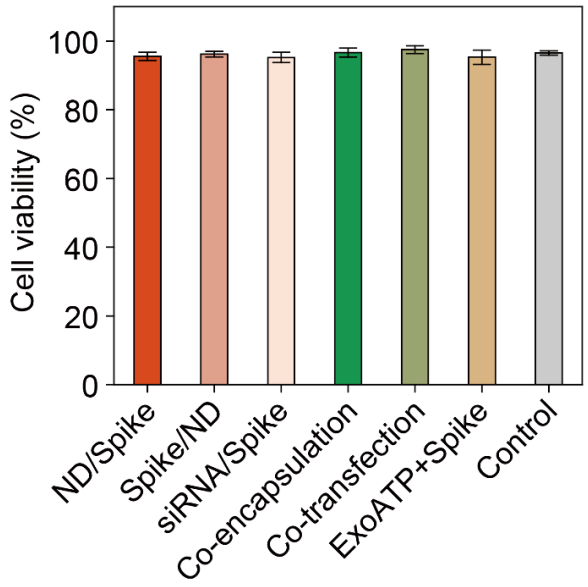


Revised Figure 5. a, Experimental design and treatment schedule. Three groups of mice are subcutaneously challenged with 5×10^5 TC1 cells/dose, followed by four intramuscular administrations of blank LNPs (control), conventional mE6-mE7 LNPs, Co-encapsulated mE6-mE7 LNPs (with ND mRNA), Co-transfected mE6-mE7 LNPs (with ND mRNA), and MIMAC-optimized mE6-mE7 LNPs, respectively. Each group contains n = 10 mice for tumor volume and survival probability measurements, with an additional n = 3 per group prepared in parallel for immunological assays using tumor and spleen samples. In each therapeutic dose, 15 ug mE6-mE7 mRNA is used. **b**, Tumor growth curves of LNP-treated mice. **c**, Survival curves of tumor-bearing mice treated with LNPs. **d**, Body weight change rates of mice treated with LNPs (normalized to Day 0). **e**, Elevated E7-specific $CD8^+$ T cell responses in spleens. Splenocytes collected on Day 23 are co-incubated with E7 Pepmix. The percentage of $IFN\gamma^+$ and $CD8^+$ T cells are quantified using flow cytometry. **f**, Elevated E7-specific $CD8^+$ T cell in tumors. Immune cells concentrated from tumors are collected on Day 23 stimulated with E7 Pepmix. The percentage of $IFN\gamma^+$ and $CD8^+$ T cells are quantified using flowcytometry. Unless stated otherwise, all LNPs are prepared using a four-component lipid formulation (ALC-0315/DSPC/Chol/PEG-lipid, 47.5/10/40.7/1.8 mol%). The N/P ratio of pre-formed LNPs is set to 6 and ND/mE6-mE7 mRNA molar ratio is set to 2. Statistical significance analysis is based on the results obtained from the experimental group and the control group: *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Illustrations are designed and licensed by Biorender.

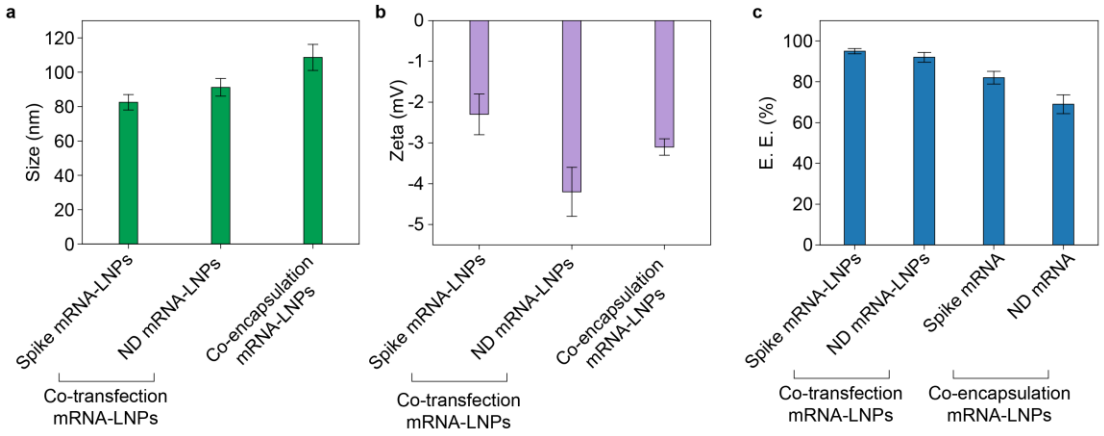


	Group	Target RNA type	Target RNA dose (μg)	ND/Target RNA molar ratio	Size (nm)
PBS	1F	NA	NA	NA	NA
Conventional	2F	linear RNA	10	0	94.8±11.5
	3F	circular RNA			90.3±12.8
	4F	saRNA	1		105.6±15.8
Co-encapsulation	5F	linear RNA	10	Uncertain	118.6±9.6
	6F	circular RNA			120.9±11.3
	7F	saRNA	1		130.4±12.1
Co-transfection	8F	linear RNA	10	2	Uncertain
	9F	circular RNA			
	10F	saRNA	1		
MIMAC	11F	linear RNA	1	2	109.9±9.2
	12F		10		
	13F	circular RNA	1		112.1±8.4
	14F		10		
	15F	saRNA	1		122.3±8.4

Revised Figure 6. a, Schematic of transformation of conventional mRNA-LNPs into self-boosting vaccines. The target linear (~1,000 nt), circular (~1,600 nt), and self-amplifying RNA (~10,000 nt) are encoded SARS-CoV-2 Omicron BA.1 Spike RBD. **b**, Group information of conventional, Co-encapsulation, Co-transfection, and MIMAC vaccines for immunization. The conventional vaccine has no ND mRNA. The Co-encapsulation vaccine has uncertain molar ratio of ND/target RNA due to stochastic assembly and the Co-encapsulation vaccine has uncertain size due to distinct LNP populations. **c**, Illustration of prime-boost immunization (Days 0 and 14) and serum IgG analysis timeline (Days 7 and 21). **d**, Anti-spike protein IgG titer post administrations. MIMAC vaccines elicit 61.3-fold (linear mRNA, 12F vs 2F), 106.9-fold (circRNA, 14F vs 3F), and 150.4-fold (saRNA, 15F vs 4F) higher titers than conventional LNPs. Low dose (1 μg) MIMAC linear and circular RNA vaccines still outperform 10 μg conventional LNPs (11F vs 2F, 13F vs 3F). **e**, Body weight changes (Day 2 vs. Day 0). High-dose conventional LNPs (10 μg, 2F, 3F, 5F, 6F, 8F, and 9F) induce weight loss (−2.0% to −6.1%) while low dose MIMAC formulations (1 μg, 11F and 13F) maintained stable weight (+0.9% and +2.0%), confirming a dose-dependent toxicity. Unless stated otherwise, all RNA-LNPs are prepared using a four-component lipid formulation (ALC-0315/DSPC/Chol/PEG-lipid, 47.5/10/40.7/1.8 mol%). The N/P ratio of pre-formed LNPs is set to 6. Six female BALB/c mice (6 weeks age) are included in each immunization group. Statistical significance analysis: *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Illustrations are designed and licensed by Biorender.



Revised Figure S16. Cell viability assessment of MIMAC mRNA-LNPs (ND/Spike, Spike/ND, and siRNA/Spike) versus conventional Co-encapsulation, Co-transfection, and ExoATP supplement strategies. Cells treated with traditional Spike mRNA-LNPs are served as the control.



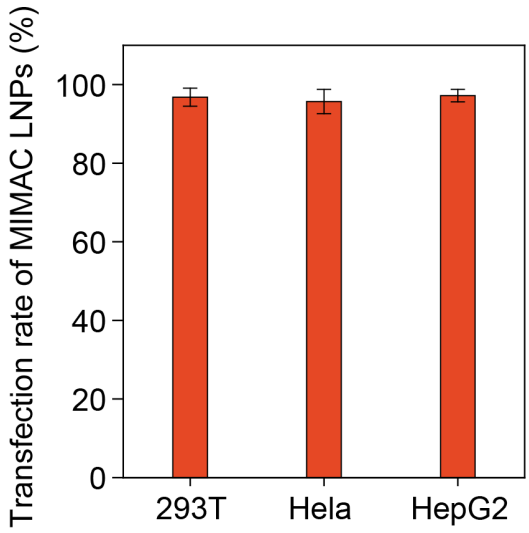
Revised Figure S17. Distinct (a), Sizes, (b), Zeta potential, and (c), E.E. of Co-encapsulation and Co-transfection mRNA-LNPs that are prepared via conventional methods.

6. The MIMAC strategy was primarily validated in 293T cells. Have the authors tested this system in other cell types to evaluate its generality? Especially, how might metabolic state differences across cell types influence the efficacy of MIMAC-formulated LNPs?

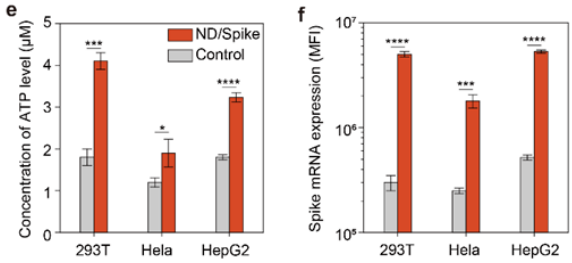
Answer: Thank you for bringing our attention to MIMAC's generalizability across cell types and metabolic states. In our new experiments, we have intentionally selected three cell lines: glycolytic-dependent HeLa (Warburg effect-prominent), oxidative phosphorylation-dominant 293T, and dual-competence HepG2 hepatocytes—thereby representing major metabolic paradigms in mammalian systems.

While MIMAC ND/Spike LNPs universally maintained transfection efficiency >95% in all the three cell lines (**revised Fig. S19**), their metabolic states critically impacted enhancement magnitude of ATP and mRNA expression: HeLa (basal ATP: 1.2 μM) exhibited attenuated responses (1.6-fold ATP↑, 7.2-fold Spike↑) vs. 293T (2.3-fold ATP↑, 16.7-fold Spike protein↑), consistent with glycolytic flux limitations; HepG2 (basal ATP: 1.9 μM) showed intermediate enhancement (1.8-fold ATP↑, 10.2-fold Spike↑), likely reflecting metabolic plasticity in transformed hepatocytes (**revised Fig. 4e-f**) .

These results confirm MIMAC's universal applicability while revealing tunable parameters for optimization. In future work, it will be worth investigating ND/therapeutic mRNA combos tailored to specific disease contexts of various metabolic signatures (e.g., cancer vs. muscular tissues). We have incorporated related modifications in the revised Manuscript (**Pages 9, 23&24**, tracking mode) and Supplementary Information (**Pages 8, 9&31**, tracking mode).



Revised Figure S19. MIMAC ND/Spike LNPs maintain high transfection efficiency (>95%) across various cell lines.



Revised Figure 4. Comparisons of **e**, Celluar ATP levels and **f**, Spike protin expression of MIMAC ND/Spike LNPs at 24 h post-transfection to Hela, 293T, and HepG2 cell lines.

7. Could the authors elaborate on the mechanism by which their metabolic rewiring strategy enhances mRNA delivery and expression? For example, a recent

study (J. Am. Chem. Soc. 2023, 145, 36, 19800–19811) demonstrated that co-delivery of ATP and mRNA improves protein expression by modulating intracellular energy availability and translation efficiency. How does the present study compare or differ from this approach, and can the authors run mechanistic studies further strengthen the hypothesis?

Answer: Thank you for offering us this opportunity to clarify the mechanistic differences between these two metabolic enhancement strategies. In response to your suggestion, we have performed new comparative studies involving co-delivery of exogenous ATP (ExoATP) with conventional Spike mRNA-LNPs (**revised Fig. 4a**). The resulting data highlight several distinct advantages of the MIMAC system, including the following key points:

(1) Mechanistically, the two approaches operate through fundamentally different routes: while the cited study (REF [34] in original Manuscript, REF [46] in revised Manuscript) showed that ExoATP improves translation, MIMAC operates by promoting endogenous ATP synthesis via ND mRNA expression.

(2) These two divergent strategies for enhancing cellular metabolism result in fundamentally divergent ND activity levels. As prompted by your earlier comment in Q4, we observed that the MIMAC strategy increased NADH dehydrogenase synthesis, which led to elevated enzyme activity (0.035 U vs. 0.017 U in controls). However, ExoATP suppressed endogenous ND activity (0.013 U vs. 0.017 U, **revised Fig. 4b**). This is because externally supplied ATP exerts feedback inhibition to upstream metabolic pathways, thereby reducing ND activity. A detailed mechanistic explanation of this distinction has been provided in our response to Q4.

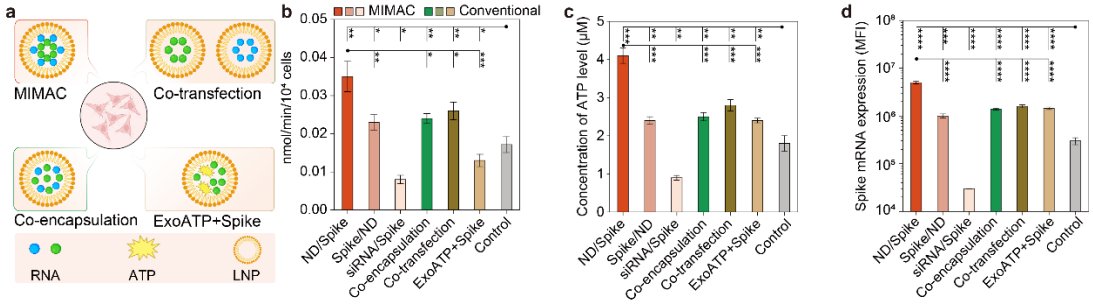
(3) The two strategies also differ markedly in both preparation process and efficacy in enhancing ATP levels. While ExoATP suffered from low encapsulation efficiency of ATP (only 20–30% retention post-formulation, as reported in the cited JACS study), MIMAC achieved >90% encapsulation efficiency for ND mRNA and generated all ATP intracellularly, thereby avoiding concerns related to insufficient encapsulation. Transfection experiments in 293T cells showed that ExoATP only increased cellular ATP levels by 1.3-fold compared to controls. In contrast, the MIMAC strategy produced a significantly stronger 2.3-fold enhancement (**revised Fig. 4c**).

(4) These differences in both ND activity and the extent of ATP elevation are directly reflected in their biological outcomes: specifically, the MIMAC system boosted target Spike mRNA expression by 16.7-fold, compared to only a 4.8-fold increase achieved by ExoATP systems at matched doses (**revised Fig. 4d**).

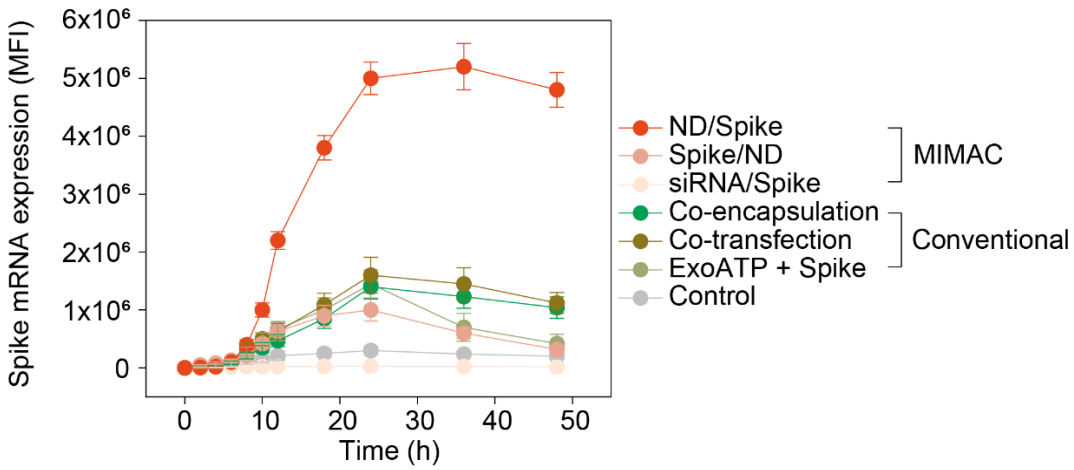
(5) Last but not least, the MIMAC platform sustained target protein expression at a plateau throughout the 24–48 h period (**revised Fig. S18**). In contrast, ExoATP produced only a transient peak at 24 h, followed by a rapid decline (>50% reduction by 36 h). This short-lived

effect can be attributed to the inefficient cellular uptake and cytosolic delivery of exogenous ATP loaded in LNPs, which limits its bioactivity. By enabling direct and continuous intracellular ATP generation, the MIMAC strategy ensures superior energy utilization and stable, long-lasting protein production.

In summary, the comparative studies clearly demonstrate that MIMAC and ExoATP delivery operate through fundamentally distinct mechanisms, with MIMAC enabling endogenous and sustained ATP production via ND mRNA expression. This strategy achieved superior ND enzyme activity and ATP levels, significantly higher target mRNA expression, and prolonged protein production compared to the transient effects of ExoATP. These advantages underline MIMAC's capacity to establish a self-sustaining bioenergetic circuit, overcoming key limitations of passive energy supplementation. All corresponding revisions, including texts, figures, and experimental details, have been thoroughly updated in the revised Manuscript (**Pages 8, 23&24**, tracking mode) and Supplementary Information (**Pages 4, 5, 8, 9&30**, tracking mode).



Revised Figure 4. a, Schematics of MIMAC and conventional co-delivery strategies. MIMAC formulations include ND/Spike (ND mRNA releasing first to boost ND), Spike/ND (Spike mRNA releasing first), and siRNA/Spike (siRNA suppressing ND releasing first); conventional groups consist of Co-transfection (dual mRNAs in separate LNPs), Co-encapsulation (dual mRNAs randomly in same LNPs), and ExoATP (Spike LNPs with exogenous ATP supplement), with Spike mRNA-LNPs alone as the control. **b**, ND enzymatic activity, **c**, Cellular ATP levels, and **d**, Spike protein expression at 24 h post-transfection of MIMAC and conventional LNPs.



Revised Figure S18. Target mRNA expression kinetics via different mRNA co-delivery strategies in HEK 293T cells.

8. This study focused primarily on local (intramuscular) administration. Have the authors also evaluated the systemic delivery of their MIMAC-formulated LNP?

Answer: We appreciate that you remind us to evaluate the systemic delivery performance of MIMAC-formulated LNPs. Pursuant to your suggestion, we have conducted intravenous (IV) studies with MIMAC LNPs encoding the ND and Omicron Spike mRNAs as SARS-CoV-2 vaccine.

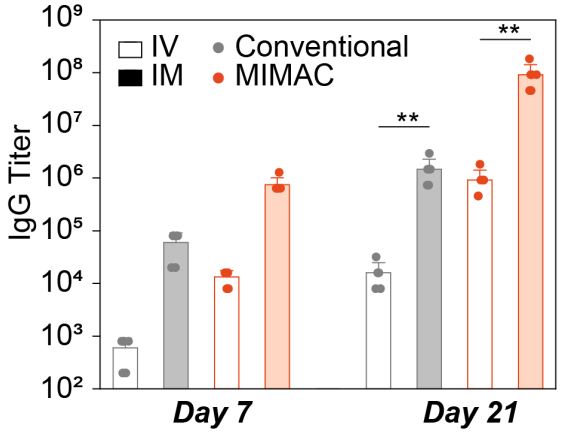
First of all, although intravenous (IV) administration of the MIMAC vaccine resulted in lower IgG production compared to intramuscular (IM) delivery after the 2nd dose (**revised Supplementary Fig. S21**), it still elicited significantly higher antibody titers than conventional vaccines ($P < 0.01$). This confirms that the MIMAC strategy remains effective across different administration routes, demonstrating its broad applicability.

Notably, IV injection was associated with greater safety concerns, which induced a 14-fold increase in IL-6 levels (**revised Supplementary Fig. S22**) and more weight loss ($P<0.01$, **revised Supplementary Fig. S23**), indicating heightened inflammatory toxicity. But these adverse effects remained within a safe and tolerable range in mice and did not cause any mortality. These results suggest that, contingent on further optimization, IV delivery of the MIMAC vaccine may still represent a promising and acceptable therapeutic option.

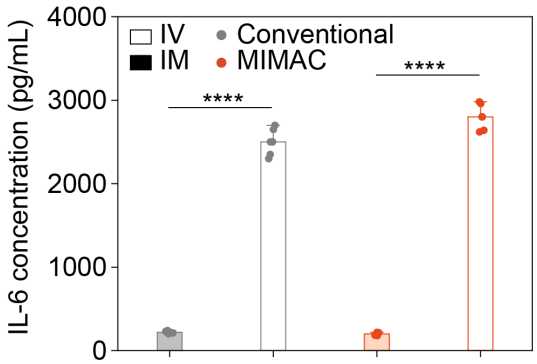
It is important to recognize that the inferior efficacy of IV versus IM administration is not specific to MIMAC but is a well-known characteristic of LNP systems, stemming from their inherent hepatotropism (**revised Supplementary Fig. S24**). This preferential hepatic sequestration of IV-injected LNPs leads to excessive antigen expression in hepatocytes and reduced delivery to immune-relevant tissues as indicated in prior studies (Pateev, Ildus, et al. *Biomedicines* 12.1 (2023): 59; Ahn, Jae-Hun, et al. *Archives of Toxicology* 99.2 (2025): 755-773; Li, Can, et al. *Clinical Infectious Diseases* 74.11 (2022): 1933-1950; Jeon, Ha-Eun, et al.

Plos one 19.10 (2024): e0311726). Given these safety risks, suboptimal immune response, and hepatotropic distribution, we were inclined not to continue with IV testing in TC1 tumor models based on animal welfare considerations.

To provide a more comprehensive evaluation while avoiding revisiting on already well-established route comparisons, we have included the comparative biodistribution data between IM and IV administration as a concise summary in the revised Manuscript (**Pages 12&13**, tracking mode) and Supplementary Information (**Pages 11&33-36**, tracking mode). We believe this additional series of experiments strengthens the rigor of our study without diverting focus from the central innovation of MIMAC. Should further investigations be needed, we would be pleased to provide the complete IV dataset or perform additional validation experiments as appropriate.

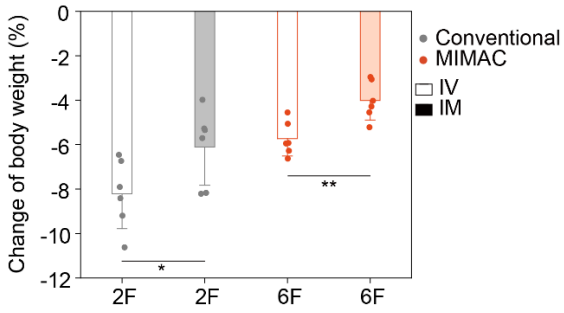


Revised Figure S21. Comparison of anti-Spike IgG antibody titers induced by conventional (encoding only Spike RBD mRNA) and MIMAC (encoding both Spike RBD and ND mRNAs) linear RNA vaccines following intravenous (IV) or intramuscular (IM) administration in mice. Hollow and solid bars stand for IV and IM injection routes, respectively. Grey and orange icons stand for conventional and MIMAC vaccines, respectively.

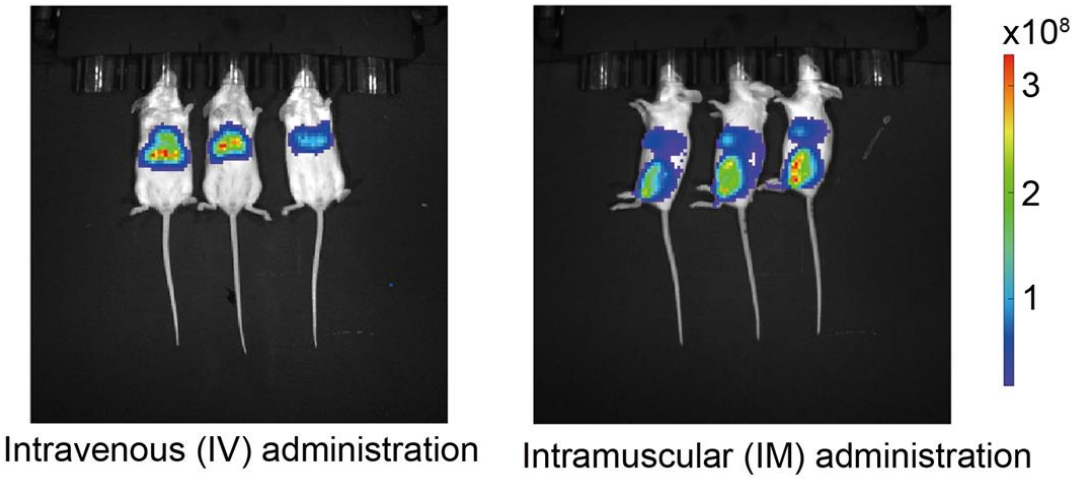


Revised Figure S22. Serum IL-6 levels in mice following intravenous (IV) or intramuscular (IM) administration of conventional (Spike RBD mRNA only) and MIMAC (Spike RBD and

ND mRNAs) linear RNA vaccines, measured on Day 21 post-immunization. Hollow and solid bars stand for IV and IM injection routes, respectively. Grey and orange icons stand for conventional and MIMAC vaccines, respectively.



Revised Figure S23. Comparison of body weight changes in mice following intravenous (IV) versus intramuscular (IM) administration of conventional (encoding only Spike RBD mRNA) and MIMAC (encoding both Spike RBD and ND mRNAs) linear RNA vaccines at Day 21 post-immunization. Hollow and solid bars stand for IV and IM injection routes, respectively. Grey and orange icons stand for conventional and MIMAC vaccines, respectively.



Revised Figure S24. In vivo imaging of intravenous (IV) versus intramuscular (IM) administrations of MIMAC LNPs (encoding Firefly luciferase and ND mRNAs) at 24 hours post-injection. IV administration results in predominant hepatic accumulation of LNPs.

Reviewer #2 (Report for the authors (Required)):

The paper reports on two main features: the use of NADH dehydrogenase-encoding mRNA in LNPs and a microfluidic-based strategy for the sequential loading of this mRNA along with another mRNA encoding a therapeutically relevant protein. The authors demonstrate that microfluidic mixing prevents aggregation (or general increases in LNP size) when compared to manual mixing methods. This is an interesting observation, though not of sufficient impact to warrant publication in Nature Biomedical Engineering.

Reply: We sincerely thank you for your thoughtful evaluation of our manuscript and for raising important points regarding the novelty and impact of our work. We appreciate the opportunity to clarify our contributions and highlight the transformative aspects of the MIMAC platform.

While we agree that the anti-aggregation property of microfluidic mixing (**Fig. 2b–c**) is a beneficial technical feature, it serves primarily as a technical tool for our core innovation: the spatiotemporal programming of mRNA delivery. The fundamental advance of MIMAC lies in its capacity to rationally engineer the internal architecture of pre-formed mRNA-LNPs, which enables:

(1) Hierarchical mRNA localization: Through precisely tuned high-frequency shear forces, MIMAC drives the insertion of secondary mRNA (e.g., ND mRNA) into the peripheral layer while simultaneously relocating the pre-encapsulated therapeutic mRNA to the core (**revised Fig. 3e–f**). This level of spatial control is unattainable via conventional Co-encapsulation (stochastic self-assembly) or Co-transfection (bulk mixing) methods.

(2) Programmable release kinetics: The stratified architecture ensures sequential release—peripheral mRNA is expressed earlier than core-localized mRNA (**revised Fig. 3g–j**). This empowers metabolic priming (via ND mRNA) before therapeutic mRNA translation, a mechanism not achievable with traditional approaches.

(3) Intracellular metabolic priming: Unlike prior studies focusing on optimizations of mRNA sequences or LNP formulations, we pioneer the use of NADH dehydrogenase (ND) mRNA as a metabolic amplifier to boost intracellular ATP levels—the central energy currency for translation. This approach directly targets the biochemical bottleneck of mRNA expression and thus reveals a new direction in mRNA therapy. By elevating ATP levels up to 4.2-fold (**revised Fig. 4g**), MIMAC achieves a 16.7-fold increase in target protein expression (**revised Fig. 4h**), significantly surpassing the efficacy of conventional Co-encapsulation, Co-transfection, and even direct exogenous ATP delivery (which suffers from ND enzymatic activity feedback inhibition and transient effects; **revised Fig. 4b–d**, **revised Fig. S18**).

This temporal control translates into significant therapeutic advantages:

- 90% tumor growth suppression in a mice HPV tumor model, accompanied by significantly enhanced antigen-specific $CD8^+$ T-cell responses (**revised Fig. 5b, e–f**).
- 62.3– to 174.8-fold increases in IgG titers against SARS-CoV-2 across linear, circular, and self-amplifying RNA platforms (**revised Fig. 6d**).
- Dose sparing (1/10 conventional dose) with reduced toxicity (**revised Fig. 6d-e**)

These results underscore that MIMAC is not merely an aggregation-control technique, but a versatile strategy for orchestrating metabolic and therapeutic gene expression within the same cell—a capability critical for mRNA or gene therapies requiring temporal coordination.

Furthermore, in direct response to your and other Reviewers' comments, we have now included several key control groups, mechanistic studies, and administration route comparisons that further validate MIMAC's superiority over conventional co-delivery methods (Co-encapsulation, Co-transfection, and exogenous ATP delivery). These additions, detailed in **revised Fig. 3–6** and **Supplementary Fig. S1**, **S14-S19**, and **S21-S24**, robustly support our claims and highlight MIMAC's unique capacity to synchronize metabolic priming with therapeutic expression.

We believe that MIMAC represents a paradigm shift in mRNA-LNP engineering, enabling previously inaccessible control over gene expression timing and synergy. Its compatibility with diverse nucleic acids (1–13 kb), lipid formulations, and administration routes, coupled with scalable production (200 doses/hour, RSD < 5%), positions it as a transformative platform for next-generation mRNA therapeutics.

Again, we are grateful for your insightful comments, which have helped us better articulate the broader implications of our work. We hope that these clarifications and additional data convincingly demonstrate the novelty, mechanistic depth, and therapeutic potential of the MIMAC strategy, aligning with the high impact standards of *Nature Biomedical Engineering*.

The concept of spatially differential loading of mRNAs into LNPs to achieve differential expression kinetics is also intriguing. However, this hypothesized difference in expression kinetics is insufficiently supported by data. Could this be better demonstrated, for example, by using two distinct fluorescent reporters?

Answer: We are particularly grateful to your insightful observation regarding the limitations in our initial experimental design. In fact, other Reviewers have also emphasized that experimental validation of the differential expression kinetics via two distinct fluorescent reporters is essential.

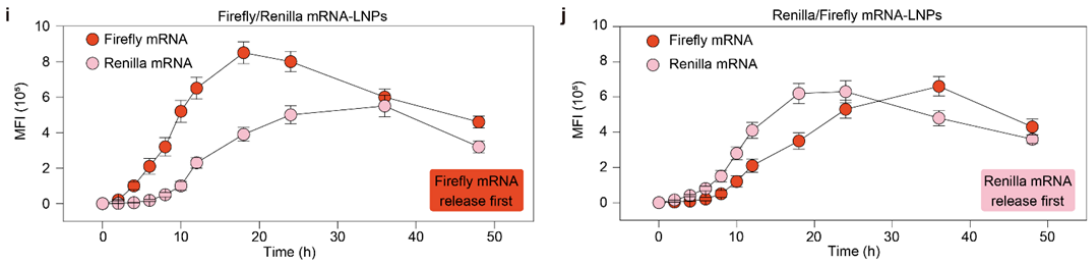
Following your suggestions, we have prepared a dual-luciferase tracking system of Firefly (F) and Renilla (R) mRNA luciferases in two different MIMAC mRNA-LNP architectures: F/R-LNPs (peripheral Firefly mRNA) and R/F-LNPs (peripheral Renilla mRNA).

Briefly, in vitro transfection of 293T cells demonstrated (**revised Fig. 3i-j**):

(1) In F/R-LNPs, peripheral Firefly mRNA exhibited significantly accelerated expression kinetics (detectable at 2 h vs. 6 h for core-localized Renilla mRNA), reaching peak expression at 18 h (vs. 36 h for core Renilla mRNA)

(2) In R/F-LNPs, peripheral Renilla mRNA showed comparably rapid translation initiation (2 h vs. 6 h for core-localized Firefly mRNA) with plateau attainment at 18-24 h (vs. 36 h for core Firefly mRNA)

These results establish that surface-localized mRNA undergoes preferential endosomal escape upon cellular uptake, thereby enabling expedited cytosolic access and translation. The staggered expression kinetics confirms MIMAC's unique capability for temporally programmable payload release. We have updated figure and textual contents in the revised Manuscript (**Page 7**, **21&22**, tracking mode) and added related protocols to the revised Supplementary Information (**Page 10**, tracking mode).



Revised Figure 3. In vitro expression kinetics of **i**, MIMAC F/R-LNPs (peripheral Firefly mRNA/core Renilla mRNA) and **j**, MIMAC R/F-LNPs (peripheral Renilla mRNA/core Firefly mRNA). Peripheral mRNA consistently demonstrates accelerated translation, with detectable expression by 2 h (vs. 6 h for core mRNA) and peak levels at 18–24 h (vs. 36 h for core mRNA).

The use of NADH dehydrogenase-encoding mRNA to metabolically activate cells with the aim of enhancing subsequent expression of the second mRNA is conceptually appealing. However, the in vivo advantage of the MIMAC strategy—versus simple co-encapsulation of the two mRNAs in aqueous medium followed by standard LNP formulation—is not demonstrated. These experiments are essential to determine whether the MIMAC strategy provides a real benefit over conventional co-encapsulation.

In view of these consideration I think this manuscript in not ready for publication

in Nature Biomedical Engineering.

Answer: We sincerely thank you for your positive recognition of the conceptual innovation of our strategy. We are also grateful for your critical comment regarding the necessity of involvement of traditional co-delivery groups in parallel evaluation, which was similarly noted by other Reviewers.

In direct response to your suggestion, we have performed comprehensive comparative studies between the MIMAC strategy and two conventional co-delivery approaches: (1) Co-encapsulation, which randomly loads ND and Spike mRNAs within the same LNPs through a single formulation step, and (2) Co-transfection, which is a mixture of separately prepared ND mRNA-LNPs and Spike mRNA-LNPs (**revised Fig. 4a**). These comparisons were conducted both in vitro and in vivo (Tumor therapy and SARS-CoV-2 prophylaxis) to rigorously evaluate the advantages of our platform.

In the in vitro studies, the MIMAC system demonstrated significant superiority. Firstly, while both conventional methods enhanced cellular ND activity (1 unit = 1 nmol NADH consumed/min/10^4 cells) relative to the control (reaching 0.024 U for Co-encapsulation and 0.026 U for Co-transfection, vs. 0.017 U for control), the MIMAC ND/Spike LNPs achieved a markedly higher enzyme activity of 0.035 U. The difference between MIMAC and either conventional method was statistically significant ($P<0.05$, **revised Fig. 4b**). Secondly, whereas conventional Co-encapsulation and Co-transfection only moderately elevated intracellular ATP levels (1.6-fold and 1.4-fold increase, respectively; $p<0.01$), MIMAC LNPs induced a significantly stronger 2.3-fold enhancement ($p<0.001$, **revised Fig. 4c**). These pronounced differences in metabolic activity directly translated into target protein expression: Co-encapsulation and Co-transfection methods improved Spike protein production by 5.3-fold and 4.7-fold, respectively, while MIMAC yielded a dramatic 16.7-fold increase over controls ($p<0.0001$ between the two conventional groups and MIMAC, **revised Fig. 4d**). We attribute these enhancements to the unique spatiotemporal control offered by the MIMAC architecture, which ensures coordinated delivery and release of distinct mRNAs into the same cell—avoiding issues such as uneven cellular uptake and resource competition inherent in Co-transfection, as well as the variable loading efficiency and non-sequential release typical of stochastic Co-encapsulation (**revised Supplementary Fig. S17**).

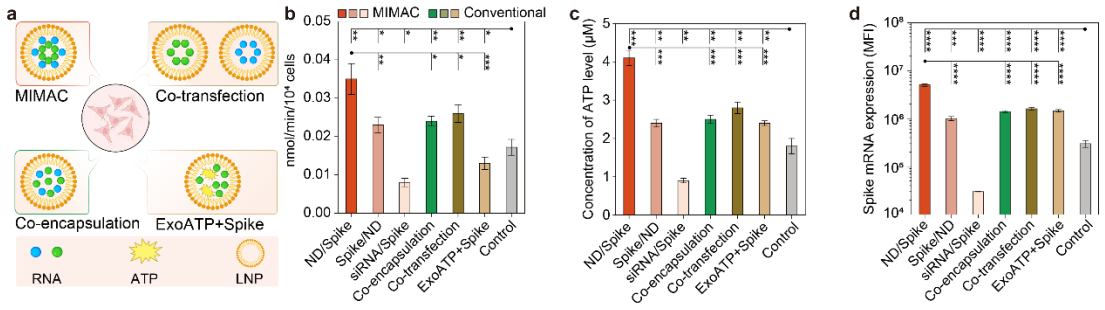
In the tumor model, the Co-encapsulation and Co-transfection groups showed intermediately enhanced efficacy compared to the conventional LNP that only contained therapeutic mE7-mE6 mRNA: Co-encapsulation achieved 74% tumor growth inhibition by Day 24 (vs. 60% in controls), while Co-transfection reached 75% inhibition. However, both groups underperformed relative to MIMAC's 90% inhibition (**revised Fig. 5b**). Survival analysis further highlighted this advantage: Co-encapsulation and Co-transfection groups retained 0% and 20% survival on Day 38 and Day 40, respectively, compared to 60% survival with MIMAC

(vs. 0% in controls on Day 35, **revised Fig. 5c**). Regarding safety profiles, both Co-encapsulation and Co-transfection groups exhibited only transient physiological perturbations, with body weight changes showing no statistically significant differences ($P>0.05$) compared to conventional mE6-mE7-LNP and MIMAC formulations (**revised Fig. 5d**). In cellular immunity assessments, mice treated with Co-encapsulation and Co-transfection formulations exhibited $IFN\gamma^+CD8^+$ T-cell frequencies of 0.54% and 0.56% in splenocytes (vs. 0.30% for conventional group) and 2.37% and 2.45% in tumor-infiltrating lymphocytes (TILs) (vs. 1.60%), respectively. These values proved statistically lower than the 0.81% (splenocytes) and 3.2% (TILs) achieved by MIMAC-treated cohorts (**revised Fig. 5e-f**).

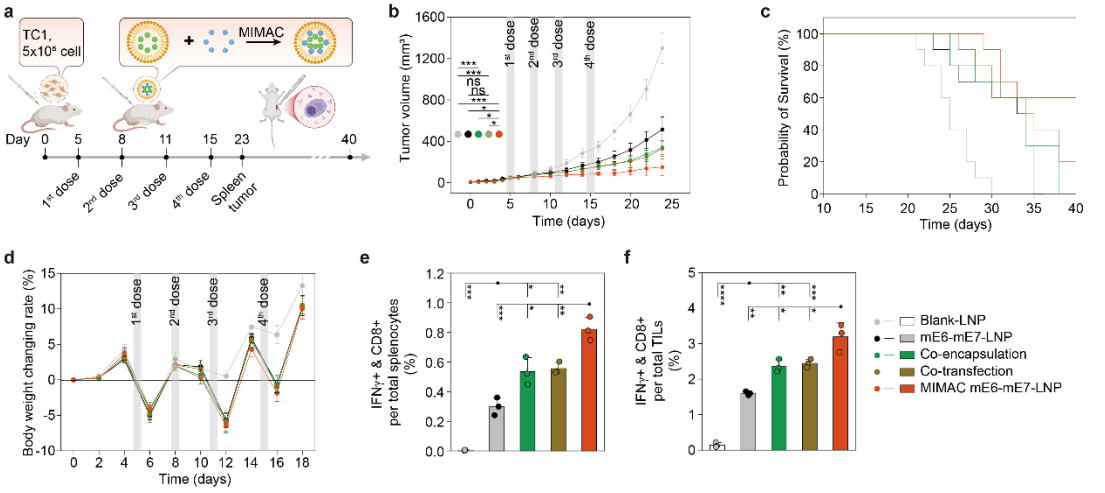
For the SARS-CoV-2 vaccine, we have now incorporated six additional cohorts: Co-encapsulation (5F-linearRNA, 6F-circRNA, 7F-saRNA) and Co-transfection (8F-linearRNA, 9F-circRNA, 10F-saRNA) with ND mRNA, alongside conventional controls containing only therapeutic RNAs (2F–4F) and MIMAC (12F–15F, **revised Fig. 6b**). Across all RNA formats (linear, circular, self-amplifying), Co-encapsulation and Co-transfection outperformed single-mRNA controls but remained inferior to MIMAC. For example, in IgG antibody titers (GMT): Co-encapsulation (5F) showed 3.20×10^6 and Co-transfection (8F) showed 3.73×10^6, respectively, whereas MIMAC exhibited 9.17×10^7 (12F, **revised Fig. 6d**). Similar trends were observed for circular and self-amplifying RNAs, with MIMAC achieving 2.70×10^8 and 3.06×10^8 GMT versus 5.46×10^7 and 6.12×10^7 GMT for Co-encapsulation/Co-transfection. These results confirm the superiority of MIMAC's spatial and temporal control over conventional mRNA co-delivery.

Collectively, the concordant findings from in vivo tumor therapy and SARS-CoV-2 vaccine studies are consistent with the in vitro results, confirming MIMAC's advantages over conventional co-delivery strategies. We attribute the advantages of MIMAC to three key factors: (1) ND mRNA enhances metabolic priming, which boosts therapeutic mRNA expression even in Co-encapsulation and Co-transfection groups compared to conventional single-mRNA controls; (2) Sequential release of ND mRNA first ensures optimal metabolic conditions before therapeutic payload delivery; (3) Although these methods have little difference in their impacts on cell viability (**revised Fig. S16**), MIMAC guarantees both mRNA co-packaging within same LNPs (superior to stochastic Co-encapsulation, **revised Fig. S17**) and synchronized delivery to same cells (addressing Co-transfection limitations, **revised Fig. S17**).

We would like to once again express our sincere gratitude for your valuable and insightful suggestions, which have greatly enhanced the rigor and impact of our manuscript. We believe that the new experimental data fully has addressed your concerns and convincingly supported our conclusions on advantages of the MIMAC platform. All corresponding revisions have been systematically incorporated into the revised Manuscript (**Pages 7–12**, **23–28**, tracking mode) and the Supplementary Information (**Pages 4**, **9**, **11**, **28–29**, tracking mode).

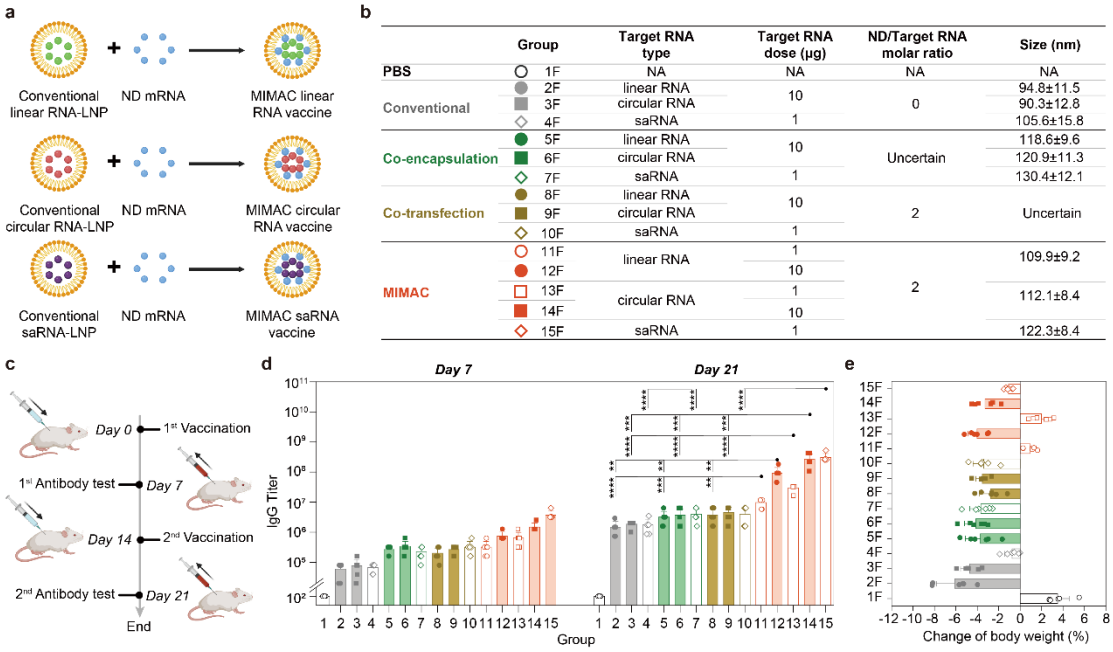


Revised Figure 4. a, Schematics of MIMAC and conventional co-delivery strategies. MIMAC formulations include ND/Spike (ND mRNA releasing first to boost ND), Spike/ND (Spike mRNA releasing first), and siRNA/Spike (siRNA suppressing ND releasing first); conventional groups consist of Co-transfection (dual mRNAs in separate LNPs), Co-encapsulation (dual mRNAs randomly in same LNPs), and ExoATP (Spike LNPs with exogenous ATP supplement), with Spike mRNA-LNPs alone as the control. **b**, ND enzymatic activity, **c**, Cellular ATP levels, and **d**, Spike protein expression at 24 h post-transfection of MIMAC and conventional LNPs.

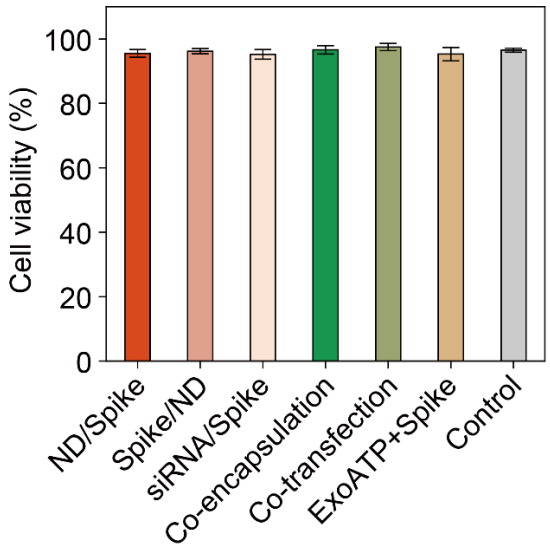


Revised Figure 5. a, Experimental design and treatment schedule. Three groups of mice are subcutaneously challenged with 5×10^5 TC1 cells/dose, followed by four intramuscular administrations of blank LNPs (control), conventional mE6-mE7 LNPs, Co-encapsulated mE6-mE7 LNPs (with ND mRNA), Co-transfected mE6-mE7 LNPs (with ND mRNA), and MIMAC-optimized mE6-mE7 LNPs, respectively. Each group contains n = 10 mice for tumor volume and survival probability measurements, with an additional n = 3 per group prepared in parallel for immunological assays using tumor and spleen samples. In each therapeutic dose, 15 ug mE6-mE7 mRNA is used. **b**, Tumor growth curves of LNP-treated mice. **c**, Survival curves of tumor-bearing mice treated with LNPs. **d**, Body weight change rates of mice treated with LNPs (normalized to Day 0). **e**, Elevated E7-specific $CD8^+$ T cell responses in spleens. Splenocytes collected on Day 23 are co-incubated with E7 Pepmix. The percentage of $IFN\gamma^+$ and $CD8^+$ T cells are quantified using flow cytometry. **f**, Elevated E7-specific $CD8^+$ T cell in tumors. Immune cells concentrated from tumors are collected on Day 23 stimulated with E7 Pepmix. The percentage of $IFN\gamma^+$ and $CD8^+$ T cells are quantified using flowcytometry. Unless stated otherwise, all LNPs are prepared using a four-component lipid formulation (ALC-0315/DSPC/Chol/PEG-lipid, 47.5/10/40.7/1.8 mol%). The N/P ratio of pre-formed LNPs is set

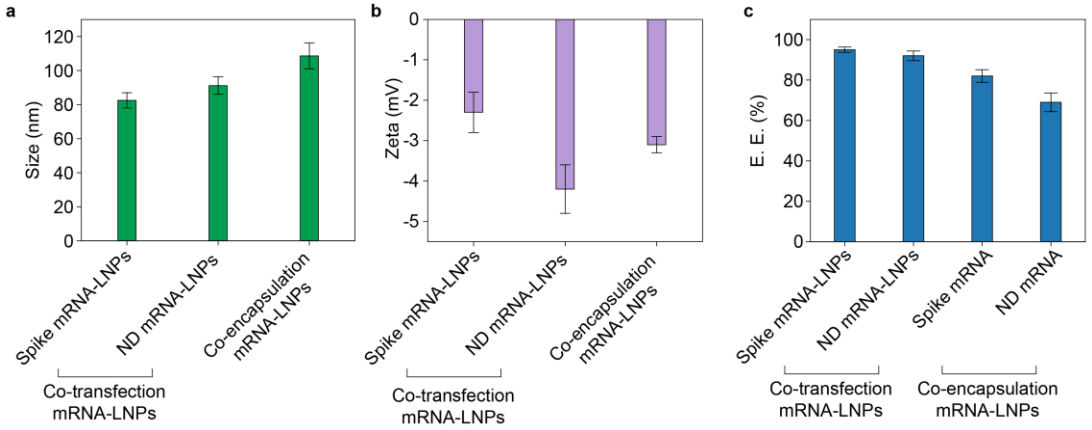
to 6 and ND/mE6-mE7 mRNA molar ratio is set to 2. Statistical significance analysis is based on the results obtained from the experimental group and the control group: *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Illustrations are designed and licensed by Biorender.



Revised Figure 6. a, Schematic of transformation of conventional mRNA-LNPs into self-boosting vaccines. The target linear (~1,000 nt), circular (~1,600 nt), and self-amplifying RNA (~10,000 nt) are encoded SARS-CoV-2 Omicron BA.1 Spike RBD. **b**, Group information of conventional, Co-encapsulation, Co-transfection, and MIMAC vaccines for immunization. The conventional vaccine has no ND mRNA. The Co-encapsulation vaccine has uncertain molar ratio of ND/target RNA due to stochastic assembly and the Co-encapsulation vaccine has uncertain size due to distinct LNP populations. **c**, Illustration of prime-boost immunization (Days 0 and 14) and serum IgG analysis timeline (Days 7 and 21). **d**, Anti-spike protein IgG titer post administrations. MIMAC vaccines elicit 61.3-fold (linear mRNA, 12F vs 2F), 106.9-fold (circRNA, 14F vs 3F), and 150.4-fold (saRNA, 15F vs 4F) higher titers than conventional LNPs. Low dose (1 μg) MIMAC linear and circular RNA vaccines still outperform 10 μg conventional LNPs (11F vs 2F, 13F vs 3F). **e**, Body weight changes (Day 2 vs. Day 0). High-dose conventional LNPs (10 μg, 2F, 3F, 5F, 6F, 8F, and 9F) induce weight loss (−2.0% to −6.1%) while low dose MIMAC formulations (1 μg, 11F and 13F) maintained stable weight (+0.9% and +2.0%), confirming a dose-dependent toxicity. Unless stated otherwise, all RNA-LNPs are prepared using a four-component lipid formulation (ALC-0315/DSPC/Chol/PEG-lipid, 47.5/10/40.7/1.8 mol%). The N/P ratio of pre-formed LNPs is set to 6. Six female BALB/c mice (6 weeks age) are included in each immunization group. Statistical significance analysis: *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Illustrations are designed and licensed by Biorender.



Revised Figure S16. Cell viability assessment of MIMAC mRNA-LNPs (ND/Spike, Spike/ND, and siRNA/Spike) versus conventional Co-encapsulation, Co-transfection, and ExoATP supplement strategies. Cells treated with traditional Spike mRNA-LNPs are served as the control.



Revised Figure S17. Distinct (a), Sizes, (b), Zeta potential, and (c), E.E. of Co-encapsulation and Co-transfection mRNA-LNPs that are prepared via conventional methods.

Reviewer #3 (Report for the authors (Required)):

This manuscript reports a microfluidic integrated mRNA amplification circuit (MIMAC). The authors prepared structured LNPs encapsulated with NADH dehydrogenase (ND) mRNA at the LNP periphery and therapeutic mRNA in the core via microfluidic shear forces. They designed and performed detailed experiments, and demonstrated the unique structure and self-boosting function of the LNP. In vivo tests of cancer immunotherapy and SARS-CoV-2 prophylaxis potentiate the applications of MIMAC derived LNP. It is an original work using ND mRNA to elevate cellular ATP level and prime metabolic pathways to amplify target mRNA translation. The manuscript is well-organized. The findings and advances in this report should have broad implications in RNA delivery and therapies.The following issues should be addressed before acceptance:

Reply: We sincerely thank you for the thorough review and highly positive feedback on our manuscript. We are particularly grateful for the recognition of the originality and potential broad implications of our MIMAC strategy in RNA delivery and therapy. We also appreciate your careful attention to detail in identifying several technical points that needed clarification or correction.

In direct response to your comments, we have made the following revisions:

- Typographical and punctuation errors (Fig. S7→S6; "0:5:1"→0.5:1) have been corrected.
- Detailed protocols for preparing oversized Cy5-labeled LNPs (> 400 nm) have been provided in the main text and Supporting Information, with reference to **revised Supplementary Fig. S13**.
- We have explicitly clarified that all comparative MIMAC samples (ND/Spike, Spike/ND, siRNA/Spike) and conventional co-delivery groups contain identical molar amounts of the respective RNAs.
- The mislabeled parameter in the Supporting Information ("frequency f" → "shear force magnitude $\boldsymbol{F}$") has been corrected.
- A thorough explanation for the use of 75°C during microfluidic processing has been provided, supported by DSC phase transition data (**revised Fig. S7**) and encapsulation efficiency optimization results (**revised Fig. S8**).

We believe these comprehensive revisions have greatly strengthened the mechanistic rigor, reproducibility, and translational relevance of our study. Thank you once again for your

constructive input, which has undoubtedly improved the quality and clarity of our manuscript.

1. page 4, line 6 from bottom, "Fig S7" should be Fig S6

2. page 5, line 18, "0:5:1" should be 0.5:1

Answer: We sincerely appreciate your thorough review of our manuscript. In response to your Q1 and Q2, we have corrected the wrong figure number and mentioned typos in the revised Manuscript (**Pages 4&5**, tracking mode).

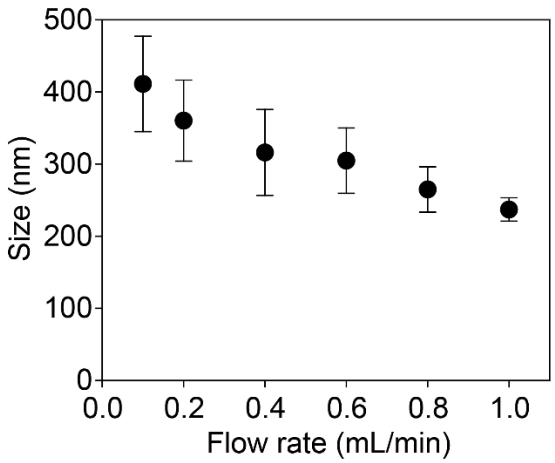
3. page 6, line 8, please provide the detail to prepare "oversized Cy5-labeled LNPs (> 400 nm)" ?

Answer: We sincerely thank you for your attention to this experimental detail. The oversized Cy5-labeled LNPs (> 400 nm) were prepared as follows:

First, DSPE-PEG_{2000}-Cy5 (Broad Pharma, BP-26203) was added into the standard four-component lipid mixture (ALC-0315/DSPC/Cholesterol/PEG-lipid = 47.5:10:40.7:1.6, mol%) at a molar ratio of 0.2% as the fluorescently labeled lipid. The mRNA of interest was dissolved in citrate buffer (pH 4.0) at a concentration of 0.2 mg/mL, while the Cy5-tagged lipid mixture was dissolved in ethanol at 15.26 mg/mL.

The two solutions were then mixed using a custom-designed microfluidic chip with a channel cross-section of 100 μm×100 μm. Mixing was performed at a total flow rate of 0.1 mL/min with an aqueous-to-organic phase flow rate ratio of 3:1. This flow rate was selected based on our empirical studies of the relationship between total flow rate and LNP size, as supported by data in **revised Supplementary Fig. S13**.

Finally, the resulting raw mRNA-LNP solution underwent buffer exchange into DPBS via ultrafiltration, yielding the final Cy5-labeled mRNA-LNPs with a controlled average size exceeding 400 nm. The detailed experimental procedure has been included in the revised Supporting Information (**Page 25**, tracking mode) and simply mentioned in the revised Manuscript (**Page 6**, tracking mode).



Revised Figure S13. Relationship of total microfluidic flow rate and diameter of mRNA-LNPs. The total flow rate is the sum of the mRNA solution (aqueous) and lipid solution (ethanol) with a flow ratio of 3:1. The concentration of mRNA is 0.2 mg/mL and concentration of of lipid is 15.26 mg/mL. The lipid formulation is ALC-0315/DSPC/Chol/PEG-lipid (47.5/10/40.7/1.8 mol%). For preparing oversized LNPs larger than 400 nm, a total flow rate of 0.1 mL/min is selected.

4. page 7, line 1-2, do both ND/Spike and Spike/ND samples have same amount of ND or Spike?

Answer: Thank you for raising this important point regarding experimental detail. Yes, both the ND/Spike and Spike/ND MIMAC samples contain identical input amounts of ND mRNA and Spike mRNA. In addition, the siRNA/Spike sample and conventional co-delivery groups have the same molar ratio of distinct RNAs (1:1). To avoid any potential confusion, we have now explicitly stated this information in both the main text (**Page 7**, tracking mode) and the corresponding figure caption (**Page 24**, tracking mode) in the revised Manuscript.

5. SI, page 4,line 10, "The frequency f" should be "the magnitude (F) of shear force"

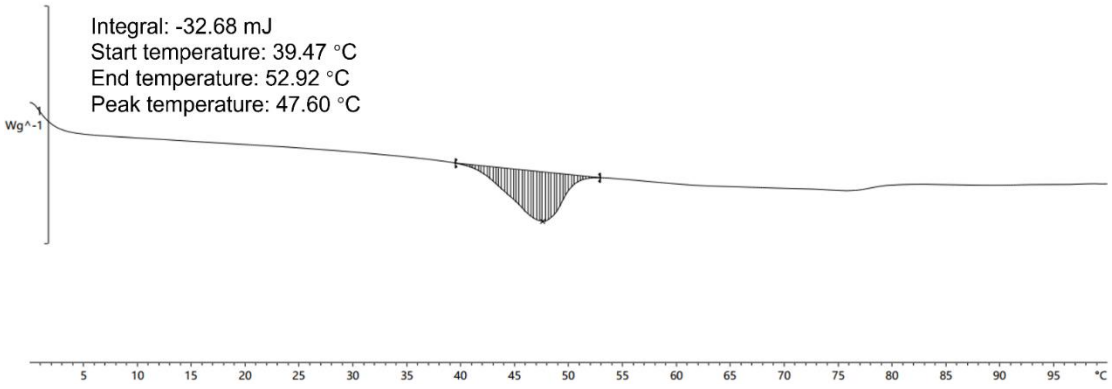
Answer: Thanks for bringing this oversight to our attention. We have corrected the description in the revised Supporting Information (**Page 5**, tracking mode).

6. please explain why the chip was set at 75oC

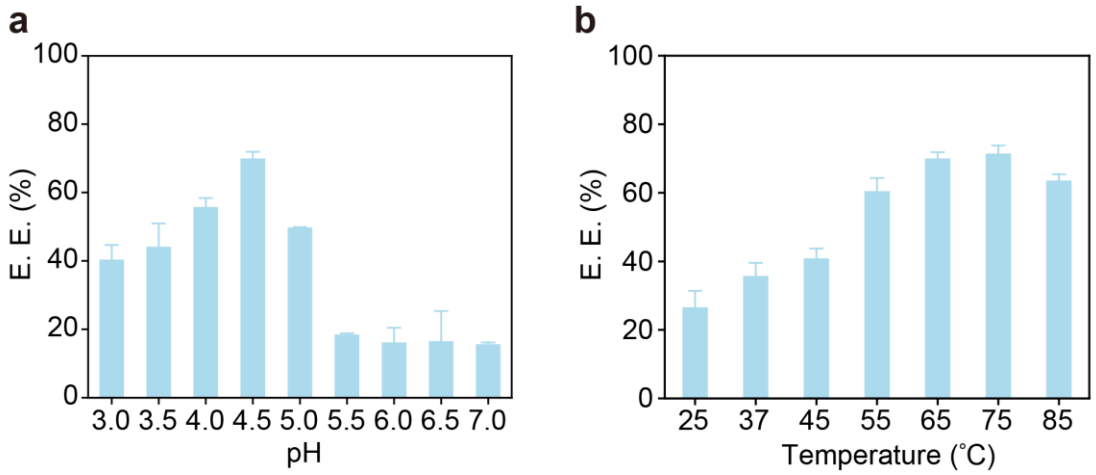
Answer: Thank you for your inquiry regarding the selected temperature for the microfluidic process. The encapsulation procedure was conducted at 75°C based on a systematic optimization of the phase behavior and encapsulation efficiency of the LNPs.

The LNPs, which are polymer-based composites, exhibit typical temperature-dependent structural properties like polymeric systems. As shown in **revised Supplementary Fig. S7**, differential scanning calorimetry (DSC) analysis indicates a broad glass transition region between 39°C and 53°C for the LNPs. Heating above this transition temperature significantly enhances lipid fluidity (Karal et al., *Soft Matter*, 19, 8285-8304, 2023) and facilitates the integration of exogenous mRNA into pre-formed LNPs.

Furthermore, as demonstrated in **revised Supplementary Fig. S8**, a temperature of 75°C is identified as optimal, resulting in the highest encapsulation efficiency of the secondary mRNA. Thus, the selection of 75°C is well-justified by both fundamental material characterization and empirical performance optimization.



Revised Figure S7. DSC results reveal that temperature-induced phase transition of LNPs occurs between 39.47 °C to 52.92 °C.



Revised Figure S8. Impacts of different (a) pH and (b) temperature on E.E. of ND mRNA in pre-formed empty LNPs in a T-junction microfluidic channel. The optimal E.E. is achieved at pH = 4.5 and T = 75 °C. The empty LNPs are prepared using a fixed four-component lipid formulation (ALC-0315/DSPC/Chol/PEG-lipid (47.5/10/40.7/1.8 mol%)). The N/P ratio is 6.