Dr. Vineeta Bajaj
Review Editor
Journal of Visualized Experiments (JoVE)
1 Alewife Center, Suite 200
Cambridge, MA 02140

December 17, 2020

Dear Dr. Bajaj,

Enclosed please find our revised manuscript "Formulating and Characterizing Lipid Nanoparticles for Gene Delivery using a Microfluidic Mixing Platform" by C.M. Bailey-Hytholt[#*], P. Ghosh[#], J. Dugas, I. Zarraga, and A. Bandekar* ([#]Co-first authors, *Corresponding authors). We thank the reviewers for their comments and suggestions and have used these to improve our manuscript (changes indicated in red within the manuscript text). The following are the details of the revisions we have made to the manuscript and our responses to the editorial and reviewer comments.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: Thank you, we have thoroughly proofread the manuscript.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: NanoAssemblr; Precision Nanosystem; Onpattro; NanoAssemblr Benchtop software; NanoAssemblr Microfluidic Cartridge; Amicon ultra centrifuge filter; Quant-iT RiboGreen reagent; Malvern Zetasizre Nano etc

Response: Thank you, we have now changed wording in the manuscript to not include these commercial product names.

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

Response: Thank you for the opportunity to fix this. All references have been updated appropriately.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of

phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response: Thank you, we have updated throughout the manuscript accordingly. We have also made more notes on when parts of the protocol should be performed inside of a biosafety cabinet.

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: Thank you, we have gone through the manuscript and have included more details. For example, we have added more detail in sections 8 and 9 with regards to measuring the LNP size, polydispersity, and zeta potential.

6. 5.7: Is there a maximum volume to be maintained?

Response: Thank you for the chance to clarify. There is no maximum volume that needs to be maintained. This has now been included in the section.

7. Please remove the embedded Tables from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. The table legend or caption (title and description) should appear in the Figure and Table Legends section after the Representative Results in the manuscript text.

Response: We have removed the Tables from the manuscript and included them in a separate file. We have added the table legend into the Figure and Table Legends section.

8. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Any limitations of the technique
b) The significance with respect to existing methods

Response: Thank you, we have now revised the Discussion section to include more focus on the technique.

Reviewers' comments:

Reviewer #1:

Major Concerns:

1. The authors should consider having more notes next to each step or a section highlighting which step is not compulsory or can be replaced without affecting the result. I think the valuable experience from the authors would make this manuscript much more useful to researchers in this field. For examples, do steps 1.1.3 and 1.2.3 necessary as the particles can be filtered to sterilize in the final step?

Response: Thank you to the reviewer for this suggestion and opportunity to add more details. We have included additional notes throughout the entirety of the manuscript. For example, we have added the following note at the end of section 1.3 regarding the buffer filtration step:

Page 3: "NOTE: Sterile filtering is highly suggested here to remove any particulates which may impact the nucleic acid quality."

2. The authors may also publish the excel spreadsheet using for calculation of the amount needed for each component, which is very useful for researchers in this field double-check/validate their calculation.

Response: Thank you to the reviewer for this suggestion. We have included detailed example calculations in sections 2 and 3 of the manuscript and have added additional notes throughout these sections that we hope will be useful for readers of the protocol. For example, we have added the following note regarding calculating the lipid volumes:

Page 4: "NOTE: When calculating the volume needed to achieve the concentration in ethanol for the microfluidic mixer, the total volume is accounted for to ensure that the addition of EtOH does not influence the lipid concentrations. For example, an ionizable lipid volume of 68.5 µL is calculated by multiplying the 5 mM concentration in ethanol by a total lipid mix volume of 533 µL and then dividing by the stock lipid concentration of 38.9 mM."

Thus, the details provided now should allow for the protocol to be successfully achieved without the need for a separate spreadsheet.

3. In Step 5, the authors used the Amicon ultracentrifuge filter to exchange buffer. This step has been reported by the Cullis group as an important process to regulate the LNPs structure (ACS Nano 2018, 12, 4787−4795) and the Culiis group used dialysis instead of ultracentrifuge filter. I think it is worthwhile to compare these two methods and comment on this point. Ultracentrifuge filter must exchange the buffer faster and more violent so would the particle size/shape be affected?

Response: We thank the reviewer for the chance to add some more clarification here. We have successfully formed LNPs using the ultra-centrifuge filters. While we have not performed a comparison between the Amicons and dialysis, we do agree that other studies have used dialysis

successfully, similar to the ACS Nano paper mentioned. We have added the following note in the manuscript:

Page 7: "NOTE: Protocol for using ultra-centrifuge filters is provided. While this method results in a more time efficient exchange of buffers, dialysis may be substituted here."

Minor Concerns:

1. The authors provide an important note that "Malic acid buffer only needs to be prepared if DNA is the nucleic acid that will be encapsulated in the LNP". I would be useful to explain the reason why the Malic acid buffer is needed. Can it be replaced by other buffers?

Response: Thank you, we have included a note in the text regarding buffer choice. While we have not explored the use of multiple different buffers for use with DNA, it is something that others might be interested in doing.

Page 3: "NOTE: We suggest citrate buffer and malic acid buffer as these have been tested and shown to be effective at successfully forming LNPs with high nucleic acid encapsulation. Other buffers may be used instead if desired. If another buffer is chosen, it is important to maintain the pH below the pKa of the ionizable lipid to ensure that the lipid is cationic and can complex with the nucleic acid."

2. The authors discussed "PEG is often used as a stealth component and can increase the circulation time for the LNPs" and "studies have investigated the acyl chain length for diffusion of PEG from the LNP, finding that short lengths (C8-14) dissociate from the LNP and are more amenable to ApoE recruitment compared to longer acyl lengths." This discussion seems to be not so relevant as the LNPs mentioned in this point encapsulate siRNA while this work focus on DNA and mRNA. I suggest the authors add a more relevant discussion on PEG branching and lipid tail length for targeted DNA delivery to lymph node citing a much more relevant work (https://doi.org/10.3390/pharmaceutics12111068).

Response: Thank you for including this useful reference for us to include. We have added additional info in the introduction to incorporate this.

Page 2: "Further, the degree of saturation of the lipid tail that PEG is conjugated to has been shown to influence the tissue distribution of LNPs[24]. Recently, Tween 20, which is a commonly used surfactant in biological drug product formulations and has a long unsaturated lipid tail, was shown to have high transfection in draining lymph nodes compared to PEG-DSPE, which largely transfected the muscle at the injection site[24]."

Reference 24: Zukancic, D., Suys, E.J.A., Pilkington, E.H., Algarni, A., Al-Wassiti, H., Truong, N.P. The importance of poly(Ethylene glycol) and lipid structure in targeted gene delivery to lymph nodes by lipid nanoparticles. *Pharmaceutics*. **12** (11), 1–16, doi: 10.3390/pharmaceutics12111068 (2020).

3. Step 1.3.2, pH was adjusted to 3. The authors may also mention an acceptable pH range.

Response: We highly suggest this pH to produce LNPs with high DNA encapsulation. We have included a note about the buffer choice including mention that an acceptable range is below the pKa of the ionizable lipid.

4. In table 1, how do the authors choose the concentrations of LNPs components in ethanol and the stocks? My group prepare the same concentration for example 10 mM of all components then just vary the volume taken to have different amounts.

Response: Thank you for the opportunity to add more details here. We have added the following note in the manuscript to clarify.

Page 4 "NOTE: Table 1 indicates stock concentrations of the lipids in ethanol. These have been shown to allow for the lipids to solubilize in ethanol, but other stock concentrations may be utilized and will not affect the outcome as long as the lipid is solubilized. Table 1 also includes example concentrations of lipids in ethanol for microfluidic mixing. These concentrations are based on the desired molar ratio, which can be varied based on the desired LNP preparation."

5. In Step 3.2 and 3.4, is it necessary to add the Avogadro's number in the equations (first multiple and then divide)? The moles of nucleic acids would be the same when deleting this number from both equations as the N/P ratio can be molar ratios and does not have to be a number ratios.

Response: Thank you for this comment. We agree that this is a preference for calculating N/P based on molecules vs. moles, but we feel that it is more thorough to show the calculation as is.

6. In step 6.3, what is the stock concentration of TritonX-100?

Response: Thank you for this question. We purchase 100% TritonX-100 and make a diluted stock of 10% (v/v). From this, we then add 2 mL of the 10% stock to 48 mL of PBS to make 50 mL buffer, which results in 0.4% (v/v) TritonX-100, which is noted in step 6.3.

7. Step 8.1, why the authors chose to dilute 50x? Would more or less dilution affect the result.

Response: Thank you, we have including the following note in the manuscript.

Page 9: "NOTE: This dilution may be changed if required. This dilution value is suggested as it uses a small volume of the LNP stock solution while providing quality results."

8. In Table 2, the flow ratio chosen was 3:1. Would this be an optimum ratio and changing this affect the LNPs formed?

Response: Thank you for the opportunity to expand here. We have added the following note in the manuscript regarding the flow ratio.

Page 6: "NOTE: A flow ratio of 3:1 and flow rate of 4-12 mL/min is suggested based on our previous studies. This has been shown to be optimal in the studies presented here, as well as by the manufacturer. This can be varied if it is of interest towards the application."

Reviewer #2:

1. In my opinion, the title should be revised as the authors discussed only about a particular microfluidic mixing device.

Response: We appreciate this suggestion and have updated the title accordingly to "Formulating and Characterizing Lipid Nanoparticles for Gene Delivery using a Microfluidic Mixing Platform."

2. Table 1, addition of excess (254uL) EtOH would change total lipid concentration? Did authors consider this issue? It wouldn't be better to take total 533uL from lipids?

Response: Thank you for the chance to expand on this and clarify. The excess EtOH does not impact the final lipid concentration as the total volume is considered when calculating. We have added a note within the manuscript to further explain.

Page 4: "NOTE: When calculating the volume needed to achieve the concentration in ethanol for the microfluidic mixer, the total volume is accounted for to ensure that the addition of ethanol (EtOH) does not influence the lipid concentrations. For example, an ionizable lipid volume of 68.5 µL is calculated by multiplying the 5 mM concentration in ethanol by a total lipid mix volume of 533 µL and then dividing by the stock lipid concentration of 38.9 mM."

3. Table 1, is it mole ratio or molar ratio?

Response: In Table 1, molar ratio is a correct usage.

4. Please check table 2; priming does not need any input for waste volumes? May be, are these parameters related to formulation 1 & 2 details?

Response: The parameters in Table 2 refer to both the priming which is described in section 4 as well as the formulation which is described in section 5. The priming does need both start and end waste, which is indicated as 0.35 and 0.05 mL, respectively.

5. In 8.3; zeta potential measurements should be done in water?

Response: Thank you for this question. Yes, indeed the zeta potential measurements are performed in nuclease free water. We have updated the text in the manuscript to be clearer.

Page 9: " 9.1 Dilute the LNP solution 40× with nuclease free water.

NOTE: Nuclease free water is used as the solvent for zeta potential measurements to minimize the influence of high salt buffers have on conductivity."

6. It is interesting and yet strange how the size of LNPs are similar for formulations prepared at 4mL/min and 12mL/min flow rates. Generally lower flow rates yield larger LNPs.

Response: Thank you for this thought. Using this technique, the manufacturer has noted that 4 mL/min is ideal for producing LNPs. Indeed, we did not observe any significant change regarding these flow rates tested for our formulations using ionizable lipid A with pDNA. It may be possible that the flow rate could have more impact on LNPs with different ionizable lipids and nucleic acids. We have added this to the manuscript.

Page 11: "This study was performed using lipid A and pDNA. It is possible that different ionizable lipids and nucleic acid could have more influence on LNP characteristics with respect to flow rate."

7. In Fig 3. the authors mentioned ~100% encapsulation of mRNA or pDNA. However, the authors did not calculate how much RNA or DNA is present inside the LNPs (as there was ~350uL waste volume during preparation). There will be significant loss of nucleic acid.

Response: Thank you for this question. The encapsulation is calculated based on the amount of mRNA or DNA inside and outside the LNPs as described in section 6. The encapsulation does not take into consideration the waste volumes during the microfluidic mixing and is based on the volume of the LNP solution after mixing. For the example calculations presented in the manuscript this is 1 mL.

8. Please mention in table 1, how much volume of LNPs will be prepared. Because in the table 1 the authors mentioned the volumes of lipids.

Response: Yes, we agree that this is helpful to include. We have now titled Table 1: "Example lipid mix to prepare 1 mL of LNPs."

9. In buffer exchange, the author should mention more common 'dialysis' method as an alternative to Amicon. Was there any difference in size between two methods?

Response: Thank you for this comment. We have not performed a side by side comparison of making LNPs using these two different wash methods, but this could be a good future study with

using the protocol provided in our manuscript. We have noted in the protocol that it is possible to substitute dialysis as an alternative to the Amicons.

Page 7: "NOTE: Protocol for using ultra-centrifuge filters is provided. While this method results in a more time efficient exchange of buffers, dialysis may be substituted here."

Reviewer #3:

Minor Concerns:

Check all the abbreviations and insert abbreviations where necessary.

Response: We have checked this and made changes in text accordingly.

Wondering if choice of buffer for exchange and storage is fixed with PBS or will it be subjective to pH of the final formulation required? Can provide clarity on the choice of buffer used in the formulation buffer exchange.

Response: Thank you for the chance to clarify. We suggest that the final buffer after the washes is PBS as it results in a pH of 7.4, making the ionizable lipid and overall LNP charge neutral. This is desirable for administering the LNPs via IV route of administration. If a different buffer or pH is desired, the investigator may change this as needed for a given application. We have included a note in the manuscript to mention the reason for PBS.

Page 7: "NOTE: PBS is chosen to increase the pH to 7.4 ± 0.2, which is physiologically relevant and will result in the ionizable lipid having a neutral charge."

We again thank the reviewers for their questions and comments and have tried to address these and use them to improve our manuscript. We feel that the updated manuscript is now suitable for inclusion in the *Journal of Visualized Experiments (JoVE)* and ask that this manuscript be considered for publication.

Sincerely,

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