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TITLE:

Formulating and Characterizing Lipid Nanoparticles for Gene Delivery using a Microfluidic Mixing Platform

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KEYWORDS:

lipid nanoparticle, nucleic acid, messenger RNA, DNA, microfluidic, staggered herringbone mixer

SUMMARY:

Lipid nanoparticles are developed using a microfluidic mixing platform approach for mRNA and DNA encapsulation.

ABSTRACT:

Lipid-based drug carriers have been used for clinically and commercially available delivery systems due to their small size, biocompatibility, and high encapsulation efficiency. Use of lipid nanoparticles (LNPs) to encapsulate nucleic acids is advantageous to protect the RNA or DNA from degradation, while also promoting cellular uptake. LNPs often contain multiple lipid components including an ionizable lipid, helper lipid, cholesterol, and polyethylene glycol (PEG) conjugated lipid. LNPs can readily encapsulate nucleic acids due to the ionizable lipid presence, which at low pH is cationic and allows for complexation with negatively charged RNA or DNA. Here LNPs are formed by encapsulating messenger RNA (mRNA) or plasmid DNA (pDNA) using rapid mixing of the lipid components in an organic phase and the nucleic acid component in an aqueous phase. This mixing is performed using a precise microfluidic mixing platform, allowing for nanoparticle self-assembly while maintaining laminar flow. The hydrodynamic size and polydispersity are measured using dynamic light scattering (DLS). The effective surface charge on the LNP is determined by measuring the zeta potential. The encapsulation efficiency is characterized using a fluorescent dye to quantify entrapped nucleic acid. Representative results demonstrate the reproducibility of this method and the influence that different formulation and

process parameters have on the developed LNPs.

INTRODUCTION:

Drug carriers are used to protect and deliver a therapeutic with typical favorable properties including low cytotoxicity, increased bioavailability, and improved stability. Polymeric nanoparticles, micelles, and lipid-based particles have previously been explored for nucleic acid encapsulation and delivery[1–4]. Lipids have been used in different types of nanocarrier systems, including liposomes and lipid nanoparticles, as they are biocompatible and effective at encapsulating nucleic acids with high stability. LNPs can readily encapsulate nucleic acids for gene delivery[5,6]. They protect the nucleic acid from degradation by serum proteases during systemic circulation[7] and can improve delivery to specific sites, as the surface topography and physical properties of LNPs influence their biodistribution[8]. LNPs also improve tissue penetration and cellular uptake[5]. Previous studies have demonstrated the success of siRNA encapsulation within an LNP[9], including the first commercially available LNP-siRNA therapeutic for the treatment polyneuropathy of hereditary transthyretin-mediated amyloidosis[10] that was approved by United States Food and Drug Administration and European Medicines Agency in 2018. More recently, LNPs are being studied for the delivery of larger nucleic acid moieties, namely mRNA and DNA[5]. As of 2018, there were ~ 22 lipid-based nucleic acid delivery systems undergoing clinical trials[10]. Additionally, mRNA containing LNPs are currently leading candidates and have been employed for a COVID-19 vaccine[11,12]. The potential success for these non-viral gene therapies requires forming small (~100 nm), stable, and uniform particles with high encapsulation of the nucleic acid.

Use of an ionizable lipid as a main component in the LNP formulation has shown advantages for complexation, encapsulation, and delivery effciciency[10]. Ionizable lipids typically have an acid dissociation constant (pKa) < 7; for example, dilinoleylmethyl-4-dimethylaminobutyrate (D-Lin-MC3-DMA), the ionizable lipid used in the FDA approved LNP formulation, has a pKa at 6.44[13]. At low pH, the amine groups on the ionizable lipid become protonated and positively charged, allowing for the assembly with negatively charged phosphate groups on mRNA and DNA. The ratio of amine, “N”, groups to phosphate, “P”, groups is used to optimize the assembly. The N/P ratio is dependent on the lipids and nucleic acids used, which varies depending on the formulation[14]. After formation, the pH can be adjusted to a neutral or physiological pH to allow for therapeutic administration. At these pH values, the ionizable lipid is also deprotonated which imparts neutral surface charge to the LNP.

The ionizable lipid also aids in endosomal escape[15,16]. LNPs undergo endocytosis during cellular uptake and must be released from the endosome in order to deliver the mRNA cargo into the cell cytoplasm or DNA cargo to the nucleus[17]. Inside the endosome is typically a more acidic environment than the extracellular medium, which renders the ionizable lipid positively charged[18,19]. The positively charged ionizable lipid can interact with negative charges on the endosomal lipid membrane, which can cause destabilization of the endosome allowing for the release of the LNP and nucleic acid. Different ionizable lipids are currently being studied for improving efficacy of both LNP distribution, as well as endosomal escape.

Other typical components of an LNP include helper lipids, such as a phosphatidylcholine (PC) or phosphoethanolamine (PE) lipid. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) are commonly used helper lipids[20,21]. DOPE has been shown to form the inverted hexagonal II (HII) phase and enhance transfection by membrane fusion[22], while DSPC has been thought to stabilize LNPs with its cylindrical geometry[23]. Cholesterol is also incorporated in the formulation in order to increase membrane rigidity, subsequently aiding in the stability of the LNP. Finally, lipid-conjugated polyethylene glycol (PEG) is included in the formulation to provide the necessary steric barrier to aid in particle self-assembly[23]. PEG also improves the storage stability of LNPs by preventing aggregation. Furthermore, PEG is often used as a stealth component and can increase the circulation time for the LNPs. However, this attribute can also pose challenges for recruitment of LNPs to hepatocytes through an endogenous targeting mechanism driven by apolipoprotein E (ApoE)[24]. Thus, 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[24]. Further, the degree of saturation of the lipid tail that PEG is conjugated to has been shown to influence the tissue distribution of LNPs[25]. 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[25]. This parameter can be optimized with dependence on the desired LNP biodistribution.

Conventional methods of forming LNPs include the thin-film hydration method and ethanol-injection method[23]. While these are readily available techniques, they are also labor intensive, can result in low encapsulation efficiency, and are challenging to scale up[23]. Advancements in mixing techniques have resulted in methods more amenable to scale up, while developing more uniform particles[23]. These methods include T-junction mixing, staggered herringbone mixing, and microfluidic hydrodynamic focusing[23]. Each method has a unique structure, but all allow for rapid mixing of an aqueous phase containing the nucleic acid with an organic phase containing the lipid components, resulting in high encapsulation of the nucleic acid[23]. In this protocol, rapid and controlled mixing through a microfluidic cartridge is utilized, which employs the staggered herringbone mixing design. This protocol outlines the preparation, assembly, and characterization of nucleic acid containing LNPs.

PROTOCOL:

A schematic of the overall process is provided in **Figure 1**.

1. Preparation of buffers

NOTE: Sterile filtering of the buffers is highly suggested here to remove any particulates which may impact the nucleic acid and LNP quality.

1.1 Phosphate Buffered Saline (PBS)

1.1.1 Prepare PBS using 8 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl in nuclease free water and adjust the pH to 7.4.

1.1.2 Sterilize by vacuum filtration using a 0.22 µm pore-size filter.

1.2 Citrate Buffer

1.2.1 Prepare citrate buffer using 5 mM sodium citrate, 5 mM citric acid, and 150 mM sodium chloride in nuclease free water and adjust to pH 4.5.

1.2.2 Sterilize by vacuum filtration using a 0.22 µm pore-size filter.

NOTE: Citrate buffer only needs to be prepared if mRNA is the nucleic acid that will be encapsulated in the LNP. If DNA will be encapsulated skip 1.2 and proceed to 1.3.

1.3 Malic Acid Buffer

1.3.1 Prepare malic acid buffer using 20 mM malic acid and 30 mM sodium chloride in nuclease free water and adjust to pH 3.0.

1.3.2 Sterilize by vacuum filtration using a 0.22 µm pore-size filter.

NOTE: Malic acid buffer only needs to be prepared if DNA is the nucleic acid that will be encapsulated in the LNP. Skip 1.3 if mRNA is to be encapsulated. Citrate buffer is used for mRNA encapsulation, as the lower pH of 3.0 with malic acid buffer may lead to an increased likelihood of mRNA degradation. The protocol can be paused here.

2. Preparation of lipid mix

2.1 If stock lipids are in powder form, solubilize in pure 200 proof ethanol.

2.2 Calculate the required mix of lipid components based on the desired molar ratio. A molar ratio of 50:10:39:1 (ionizable lipid:helper lipid:cholesterol:PEG) will be used here as an example for a total lipid concentration of 10 mM. **Table 1** shows the concentrations and volumes needed for each of these components.

NOTE: When calculating the volume needed to achieve the lipid mix concentration in ethanol (EtOH) 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.

2.3 Add the appropriate amount of each lipid stock solution to a glass vial to allow components

to mix with intermittent gentle swirling. Add 200 proof ethanol for a total mixture of 533 µL. For the example in **Table 1**, this is 254 µL of ethanol.

NOTE: For a single run to produce 1 mL of LNPs, 342.5 µL of lipid solution is needed. This is due to a 3:1 mix of aqueous nucleic acid to organic lipid solution with some volume discarded before and after sample collection. A mix of 533 µL is made to compensate as overage.

3. Preparation of nucleic acid solution

NOTE: Preparation and handling of nucleic acid solutions is to be performed in a sterile and RNase-free environment wherever possible. Work in a biosafety cabinet whenever possible with the nucleic acid.

3.1 Calculate N/P ratio. The N/P ratio is the total number of ionizable lipid amine groups (N) to the total number of negatively charged nucleic acid phosphate groups (P). N/P ratio is often a parameter that can be optimized during LNP formation. Follow the steps below.

3.1.1 Calculate the number of N units using the below formula:

$$N = Ionizable\ lipid\ concentration \times Lipid\ mix\ injection\ volume \times Avogadro's\ number \times Number\ of\ N\ per\ ionizable\ molecule$$

NOTE: The ionizable lipid concentration (**Table 1**) is 5 mM, which is equivalent to 5 x 10^{-6} mol/mL. The required lipid injection volume is 0.3425 mL. For example, if the number of N units per molecule is 1, using the above equation, there are 1.03 x 10^{18} N units in the lipid mix.

3.1.2 Calculate the P units for the desired N/P ratio. Here an N/P = 36 is used for example.

$$P = \frac{N}{36} = \frac{1.03 \times 10^{18}}{36} = 2.86 \times 10^{16}$$

3.2 Calculate the necessary nucleic acid concentration to obtain 2.86 x 10^{16} P units using the below equation.

$$Moles\ of\ nucleic\ acid = \frac{P}{Avogadro's\ number \times Number\ of\ P\ per\ base\ pair \times Number\ of\ bases}$$

Where, the number of P units per base pair for mRNA is 1 and DNA is 2. For an mRNA with 1,200 bases, the amount of mRNA required for a N/P = 36 is 3.96 x 10^{-11} moles.

3.3 Calculate the mass concentration of mRNA required for N/P = 36 using the below equation.

$$Mass\ concentration\ of\ mRNA = \frac{Moles\ of\ nucleic\ acid\ \times Molecular\ weight\ of\ nucleic\ acid}{Nucleic\ acid\ solution\ injection\ volume}$$

The average molecular weight of a ribonucleotide monophosphate unit is 322 g/mol[26]. With 1,200 base mRNA, the molecular weight of the mRNA is 386,400 g/mol. The required injection volume of nucleic acid solution is 1.028 mL. Thus, the concentration of mRNA needed is 1.488×10^{-5} g/mL, which is 14.88 µg/mL.

3.4 Make up 1.5 mL of 14.88 µg/mL concentration of mRNA in citrate buffer.

NOTE: When DNA will be the nucleic acid encapsulated, use malic acid buffer to make up the nucleic acid solution.

4. Priming the microfluidic channels

NOTE: This protocol is adapted from the instrument manufacturer's guidelines.

4.1 Input the priming parameters into the instrument software by clicking on the appropriate fields (**Table 2**).

NOTE: A flow ratio of 3:1 and flow rate of 4-12 mL/min is recommended[23,27] . 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.

4.2 Open the instrument lid and place a microfluidic cartridge into the rotating block.

4.3 Draw at least 0.5 mL of ethanol into a 1 mL syringe, ensuring there are no bubbles or air gaps at the syringe tip. Load this syringe into the right inlet of the cartridge.

4.4 Fill a 3 mL syringe with 1.5 mL of aqueous buffer (citrate for RNA and malic acid for DNA), ensuring there are no air bubbles or gaps. Load this syringe into the left inlet of the cartridge.

4.5 Insert two 15 mL conical tubes in the clip holders to serve as waste containers.

4.6 Click on **Run** in the instrument software to begin the mixing, ensuring that the parameters are input correctly.

4.7 When the instrument stops priming, indicated by the bottom blue light shutting off, open the lid and properly dispose of the conical tubes and syringes.

5. LNP formation

NOTE: This protocol is adapted from the instrument manufacturer's guidelines.

5.1. Update the software with the formulation parameters by clicking on the appropriate fields (**Table 2**).

5.2 Fill a 1 mL syringe with the lipid mix (prepared in step 2). Remove any air gaps or bubbles at the syringe tip and insert the syringe into the right side of the cartridge.

5.3 Draw the nucleic acid solution (prepared in step 3) into a 3 mL syringe, ensuring there are no bubbles or air gaps in the syringe tip. Insert the syringe into the left inlet of the cartridge.

NOTE: Volumes are provided to make a 1 mL solution of LNPs. This instrument can incorporate syringe sizes up to 10 mL, and volumes can be scaled accordingly with no influence on the outcome. The maximum volume of LNPs that can be prepared in one preparation is 12 mL.

5.4 Label a 15 mL RNase free conical tube with the sample name and insert into the left tube clip. Place a 15 mL waste conical in the right tube clip.

5.5 Close the instrument lid and click **Run**, after confirming correct input of parameters.

5.6 After the instrument is finished running, properly discard the waste container and cartridge. Retain the conical tube with the LNP sample.

5.7 Dilute the LNP 5x with PBS to minimize the ethanol to <5% (v/v).

NOTE: It is important to dilute the LNPs in PBS as soon as possible after microfluidic mixing to prevent degradation. Always perform the dilution in a biosafety cabinet and continuing to work in the biosafety cabinet throughout the buffer exchanges.

6. Buffer exchange

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.

6.1 Pre-wash an ultra-centrifuge filter (100 kDa pore size) with 2 mL of PBS by centrifuging at 1000 x *g* for 5 min. Empty the PBS from the bottom compartment.

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.

6.2 Add diluted LNPs to the top compartment of the pre-washed ultra-centrifuge filter and centrifuge at 1000 x *g* for 12 min.

6.3 Discard the flow-through from the bottom compartment. Perform two more washes by adding 5 mL of PBS to the ultra-centrifuge filter each time. Centrifuge at the same parameters. There is no maximum volume that needs to be maintained.

NOTE: If a scaled-up volume of LNPs were prepared, increase the volume of PBS for each wash accordingly. For example, if 2 mL of LNPs were prepared in a single run, then 10 mL PBS per wash is suggested.

6.4 Pipette the LNP solution against the walls of the ultra-centrifuge filter a few times to minimize LNP loss. Remove the LNP solution from the ultra-centrifuge filter and store in a nuclease free vial. Add PBS if needed to achieve a final volume of the LNP solution of 1 mL.

6.5 Filter through a pre-wet 0.2 µm syringe filter, if needed.

NOTE: The protocol can be paused here.

7. Measure encapsulation efficiency

7.1 Prepare a standard curve by making 2-fold serial dilutions of working nucleic acid solution in PBS, starting with a highest concentration of 500 ng/mL, and making at least five dilutions. Use PBS as a blank.

7.2 Prepare the LNP sample dilutions. Dilute LNP samples with PBS, to achieve an approximate theoretical concentration that lies around the mid-point of the standard curve (eg. ~ 250 ng/mL of nucleic acid estimated from the initial concentration).

7.3 Prepare a solution of the RNA quantification reagent (for mRNA measurements) with TritonX-100 to disrupt the LNPs and measure the total amount of nucleic acid inside and outside of the LNP. This solution contains 0.5% (v/v) RNA reagent, 0.4% (v/v) TritonX-100, and 99.1% (v/v) PBS.

7.4 Prepare a solution of the reagent without TritonX-100 to measure the amount of nucleic acid not encapsulated in the LNPs. This solution contains 0.5% (v/v) RNA reagent and 99.5% (v/v) PBS.

NOTE: If LNPs encapsulate double stranded DNA (dsDNA), such as plasmid DNA, use the dsDNA reagent in 6.3 and 6.4 instead, following the same procedure.

7.5 In a 96-well black fluorescence capable plate, load at least four replicates of each of the LNP and nucleic acid standard solutions prepared in 6.1 and 6.2.

7.6 To half of the replicates of standards and samples, add an equal volume of the reagent containing TritonX-100. This will quantify the total amount of nucleic acid.

7.7 To the remaining wells of standards and samples, add an equal volume of the reagent without

TritonX-100. This will quantify the amount of nucleic acid not encapsulated inside the LNP.

7.8 Shake the plate for 5 min at room temperature to ensure thorough mixing of standards and samples with the added reagent, taking precautions to avoid light exposure.

7.9 Measure the fluorescence using a microplate reader, with an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

7.10 Calculate the concentration of nucleic acid outside of the LNP using the standard curve made with the addition of the reagent without TritonX-100. Multiply by the dilution factor used in 6.2.

7.11 Calculate the concentration of nucleic acid both inside and outside of the LNP using the standard curve made with the addition of the reagent containing TritonX-100. Multiply by the dilution factor used in 6.2.

7.12 Calculate the concentration of nucleic acid inside by subtracting the concentration of nucleic acid outside (calculated from step 6.9) from the total concentration of nucleic acid both inside and outside (calculated from step 6.10)

7.13 Quantify the encapsulation efficiency from the ratio of the concentration of nucleic acid inside the LNP (calculated from step 6.11) and the total concentration of nucleic acid (calculated from step 6.10).

NOTE: The protocol can be paused here.

8. Concentration adjustments

8.1 If needed, adjust the nucleic acid concentration within the LNP solution using the results from the encapsulation efficiency.

8.2 If a less concentrated solution is desired, dilute the solution with PBS to achieve the desired concentration.

8.3 If a more concentrated solution is desired, perform additional centrifugation runs using an ultra-centrifuge filter.

NOTE: The protocol can be paused here.

9. Measure LNP hydrodynamic size and polydispersity

9.1 Dilute the LNP solution 40x with PBS.

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.

9.2 Using a semi-micro cuvette, measure the hydrodynamic diameter and polydispersity index. Add the LNP solution into the cuvette and insert into the instrument. Set up an operating procedure in the instrument software to include the measurement type, sample details (material, dispersant, temperature, and cell type), and measurement instructions (number of runs). Click **Start** when ready to begin the measurement acquisition.

10. Measure LNP zeta potential

10.1 Dilute the LNP solution 40x 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 on conductivity.

10.2 Using a folded capillary zeta cell, measure the zeta potential.

10.2.1 Add the LNP solution into the cuvette up to the fill line. Insert into the instrument ensuring that the electrodes are making contact with the instrument.

10.2.2 Set up an operating procedure in the instrument software to include the measurement type, sample details (material, dispersant, temperature, and cell type), and measurement instructions (number of runs). Click **Start** when ready to begin the measurement acquisition.

REPRESENTATIVE RESULTS:

Multiple batches of LNPs with the same lipid formulation and N/P ratio of 6 were developed on separate days to demonstrate reproducibility of the technique. Batch 1 and 2 resulted in overlapping size distributions with similar polydispersity (**Figure 2A**) No significant difference was observed in the size or encapsulation efficiency between the two different batches (**Figure 2B**). The encapsulation efficiency was high for each batch (>98.5%) and the sizes were similar with a 77 nm LNP diameter. The particles were uniform with an average polydispersity index (PDI) of 0.15 for batch 1 and 0.18 for batch 2.

Changes in formulation parameters showed some small, yet statistically significant differences with respect to the N/P ratio, ionizable lipid used, and nucleic acid encapsulated. While differences are discussed, it is important to note that all LNPs formed resulted in encapsulation greater than 80%, with most formulations greater than 95%, and particle sizes less than 110 nm, making all formulations developed here desirable for gene delivery. First, ionizable lipid A was used to develop LNPs at an N/P of 10 and 36. Decreasing the N/P ratio resulted in a 4% decrease in encapsulation efficiency and an increase in the hydrodynamic diameter of the LNPs from 98 nm at N/P = 36 to 109 nm at N/P = 10 (**Figure 3A**). Comparing LNPs with ionizable lipid A to a different ionizable lipid B and maintaining N/P of 36 resulted in a significant change in encapsulation efficiency, where 100% of pDNA was encapsulated with LNPs formed using ionizable lipid A and 81% of pDNA was encapsulated with LNPs formed using ionizable lipid B (**Figure 3B**). Ionizable lipid B LNPs also resulted in slightly smaller particles with a hydrodynamic

diameter of 95 nm. Finally, LNPs were formed using ionizable lipid A with both mRNA and pDNA. LNPs encapsulating pDNA resulted in larger particles with a 119 nm diameter compared with mRNA LNPs with a 91 nm diameter (**Figure 3C**). Both pDNA and mRNA LNPs resulted in similar encapsulation efficiency at ~91-94%.

Lastly, changes in the flow rate process parameter did not impact the LNPs developed at the flow rates tested here. At both 4 mL/min and 12 mL/min, LNPs were developed and characterized to have encapsulated 96% of pDNA and have a 110 nm diameter (**Figure 4**). All LNPs regardless of process parameter or formulation parameter resulted in charge neutral zeta potential measurements.

FIGURE AND TABLE LEGENDS:

Figure 1: LNP development and characterization workflow. First, lipid mix and nucleic acid solutions are made (**1** and **2**). The lipid mix contains the ionizable lipid, helper lipid, cholesterol, and PEG in ethanol, while the nucleic acid solution contains either mRNA or DNA in buffer. Solutions are mixed using a microfluidic cartridge (**3**), which forms LNPs (**4**). Next, a buffer exchange is required to remove the ethanol and increase the solution pH to neutral (**5**). Characterization of LNPs is performed to determine encapsulation efficiency and particle size, polydispersity, and zeta potential using a fluorescence microplate assay and zetasizer, respectively (**6** and **7**).

Figure 2: Batch to batch reproducibility of LNPs formed on separate days. (**A**) Size distributions for batch 1 vs. batch 2 (**B**) Encapsulation efficiency (%) and hydrodynamic diameter (nm) for each batch with mRNA and N/P = 6. Error bars note standard deviation. Statistical analysis using two-way ANOVA with $\alpha = 0.05$ shows no significance.

Figure 3: Variations of formulation parameters. (**A**) LNPs formed at N/P = 10 and 36 both using ionizable lipid A with pDNA. (**B**) LNPs formed with ionizable lipid A and ionizable lipid B both at N/P = 36 with pDNA. (**C**) LNPs formed with either mRNA or pDNA both using ionizable lipid C at N/P = 6. Error bars note standard deviation. Statistical analysis was performed using two-way ANOVA with $\alpha = 0.05$; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Figure 4: Variations of process parameters. LNPs formed at a flow rate of 4 and 12 mL/min using ionizable lipid A with pDNA at N/P = 10. Error bars note standard deviation. Statistical analysis using two-way ANOVA with $\alpha = 0.05$ shows no significance.

Table 1: Example lipid mix to prepare 1 mL of LNPs. The lipid stock concentrations in ethanol provided 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. Example concentrations of lipids in ethanol for microfluidic mixing are also provided. These concentrations are based on the molar ratio, which can be varied based on the desired LNP preparation.

Table 2: Microfluidic Mixing Benchtop Instrument Software Priming and LNP Formulation Example Parameters

DISCUSSION:

Reproducibility, speed, and low volume screening are significant advantages of using microfluidic mixing to form LNPs compared to other existing methods (e.g., lipid film hydration and ethanol injection). We have demonstrated the reproducibility of this method with no impact on encapsulation efficiency or particle size observed with different LNP batches. This is an essential criterion for any therapeutic, including LNPs, to become clinically available.

The technique described here employs staggered herringbone microfluidic mixing, which results in LNP formation on the time scale of only a few minutes. This mixing uses chaotic advection which is advantageous for mixing control and shortened time[23]. This mixer enables the aqueous and organic phases to effectively wrap around each other[23]. Using the staggered herringbone mixing, previous studies have shown that the particles form at the smallest thermodynamically stable size[28], which means that the composition tends to influence the size and polydispersity of the LNPs[23,28,29]. This was observed in the representative results, where the N/P ratio, ionizable lipid used, and nucleic acid encapsulated were the impacting factors on changes in encapsulation efficiency and particle size. Operating parameters, such as flow rate and ratio of mixing can also influence the size above a certain threshold, where afterwards the particle size is at its smallest stable size[23, 29]. No change in encapsulation efficiency or particle size was observed when a flow rate of 4 mL/min vs. 12 mL/min was used. Thus, likely both flow rates are above the threshold that would impact the LNP outcome. The example experiment, and results described above used 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. Other types of microfluidic mixing include the T-junction, which uses turbulent flow and the microfluidic hydrodynamic focusing method that is based on convective-diffusive mixing[23]. Compared to these other types of microfluidic mixing techniques for LNP development, the staggered herringbone mixing enables the combination of three important criteria: rapid mixing, minimizing batch to batch variability, and is commercially available[23]. All three of the microfluidic mixing methods do allow for higher encapsulation efficiency and controlled size compared to conventional lipid film hydration or ethanol injection methods[23].

Finally, the ability to produce low volumes for producing various LNP formulations at the research & development stage is a significant advantage. One challenge of developing LNPs is the number of variables that can be tested and optimized per formulation to achieve the desired outcome and efficacy. Lipids and nucleic acids can be cost prohibitive to screen, troubleshoot, and modify many formulation parameters (e.g., molar ratios, N/P ratios, process parameters, etc.) to find the most suitable LNP for a given application. While low volumes could be a limitation for producing a final formulation at a large scale, the ability to scale up the technique with larger microfluidic mixing instruments is commercially available.

Critical steps of the protocol start with proper storage of lipid stock solutions at the

manufacturer's recommendation. LNPs should then be stored at 2-8 °C until further use. For the nucleic acid preparation, the results presented demonstrate that citrate buffer and malic acid buffer are effective at successfully forming LNPs with high nucleic acid encapsulation[30, 31]. 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. When using the microfluidic mixing instrument, it is important to prime the cartridge prior to LNP formation, not to exceed the use a cartridge as recommended by manufacturer, and to change the cartridge in between different formulation compositions. The most common flow ratio for formation of the aqueous: organic solution is 3:1; however, this can be changed if needed. The flow rate can also be adjusted as desired. Finally, it is important when working with mRNA to ensure an RNase free environment throughout the entire process. If the desired size or encapsulation efficiency is not achieved, some places to begin troubleshooting include changing the N/P ratio used or the lipid molar percentages. The instrument process described here uses a benchtop model that has a maximum volume limit of 12 mL, although this process is scalable to larger volumes using different microfluidic mixing models. This process can be adapted to changes in lipid mixtures and nucleic acids for use in developing LNPs for various clinical indications. With this flexibility, numerous future applications can be achieved with LNPs to produce different desired formulations. This technique has also been used for developing other types of nanoparticles, including liposomes and polymeric nanoparticles. With some parameter changes, this method can be used for a variety of nanoparticle formulations.

The protocol detailed here describes a reproducible method for achieving mRNA or DNA encapsulated LNPs. In addition to process parameters, additional considerations can influence the LNP outcome. Previous work has also used similar methods to produce LNPs with various nucleic acids, ionizable lipids, N/P ratios, PEG linker length, etc. These parameters can influence the encapsulation efficiency, size, and charge of the particles. The instrument manufacturer has also noted similar changes depending on these parameters that can be optimized[14,32]. These parameters can further influence the biodistribution and efficacy of the nucleic acid. For example, studies have investigated hydrocarbon chain lengths (C14, C16, and C18) conjugated to PEG and found that the shorter acyl chain of C14 resulted in higher levels of liver uptake compared to the longer acyl chain, which remained in circulation for a longer period of time[24]. This protocol allows for formation of LNPs with varied compositions to optimize and test for, which makes this a versatile process.

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DISCLOSURES:

All authors are employees of Sanofi. The authors declare that they have no conflict of interest or competing financial interests.

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Figure 1

Click here to access/download;Figure;Figure 1 JoVE protocol diagram.pdf

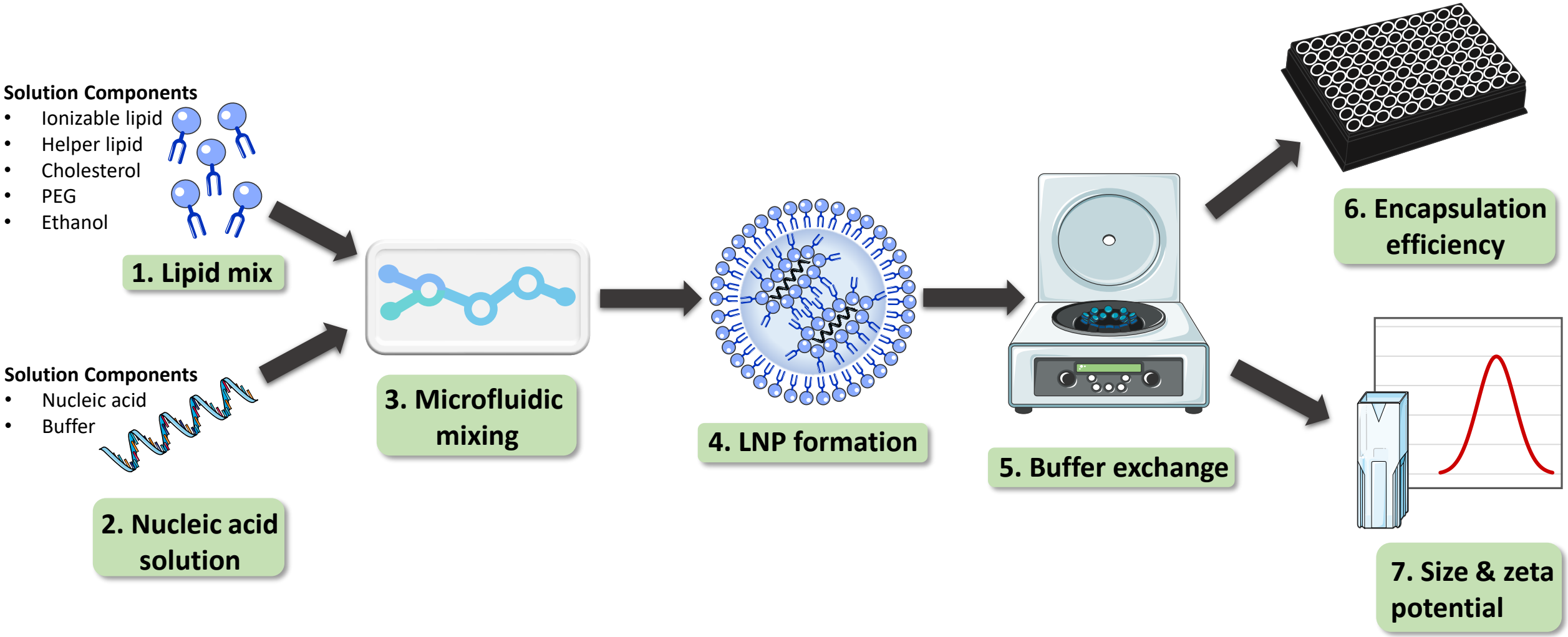

Figure 2

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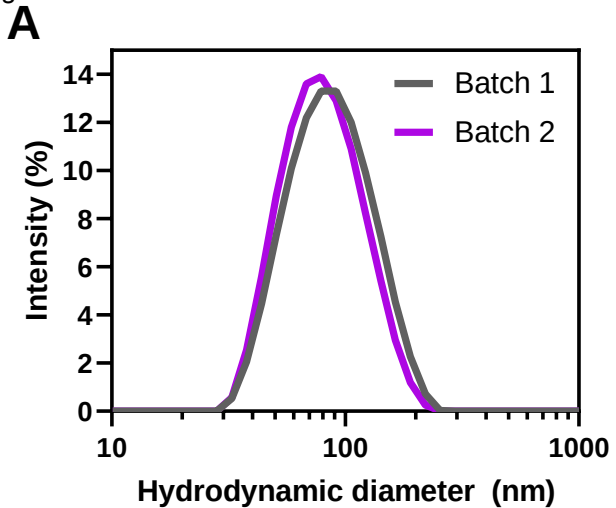

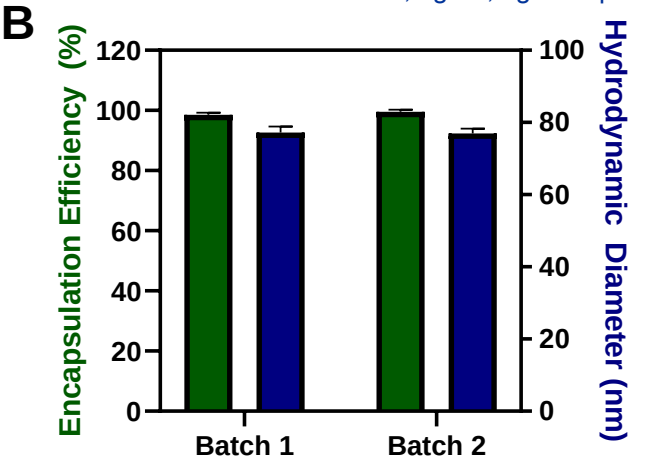

Figure 3

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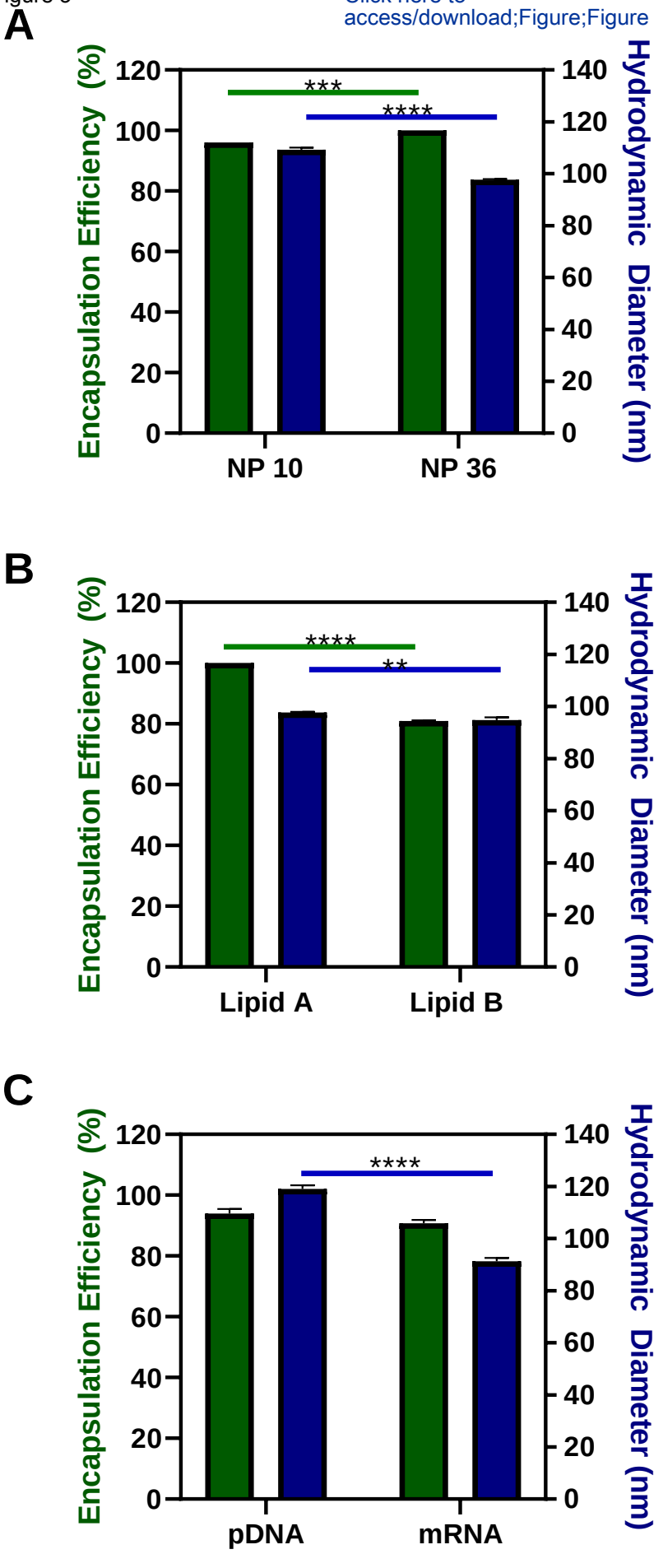

Figure 4

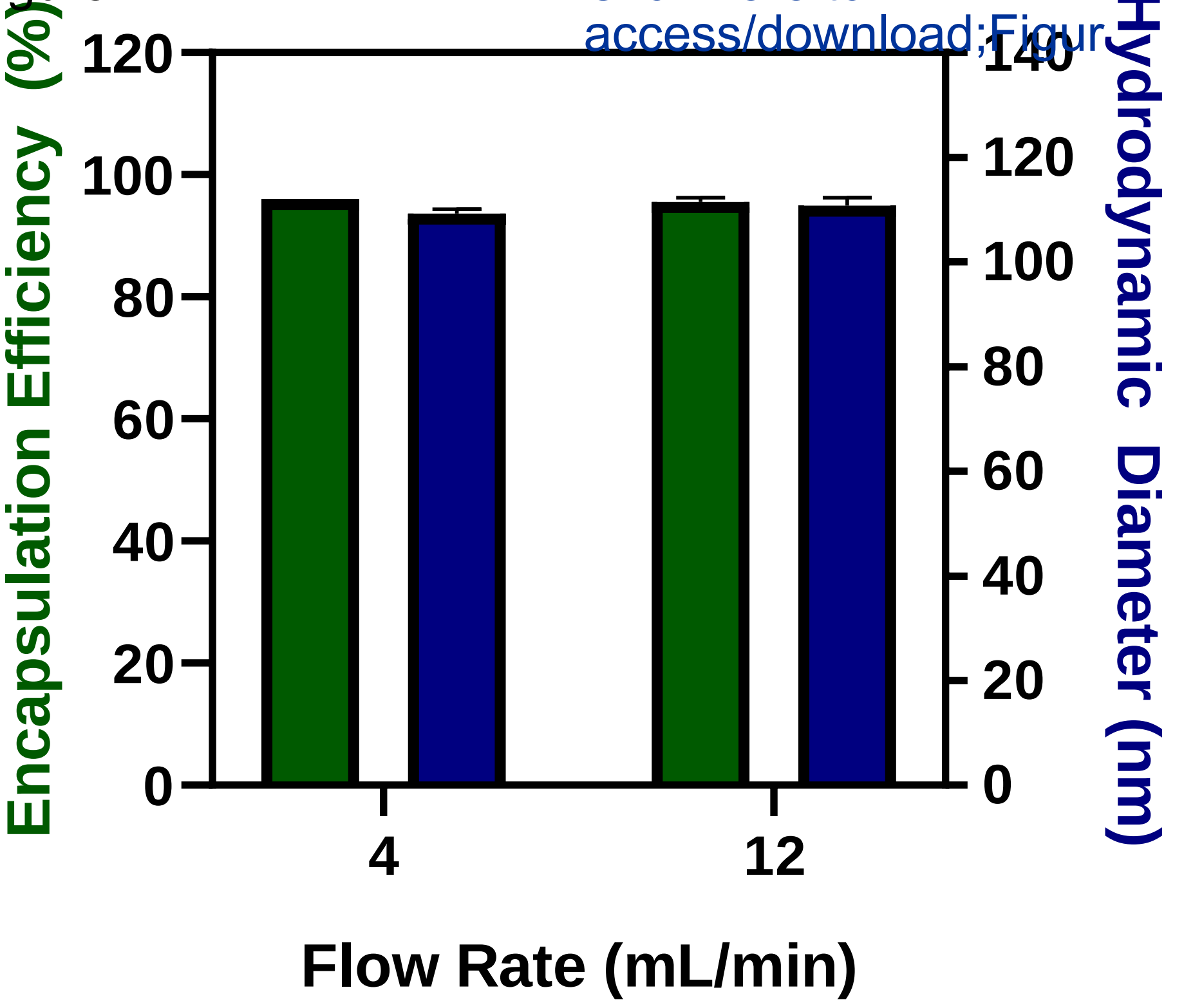

Table 1

Lipid	Molar Ratio	Stock lipid concentration (mM)	Concentration in ethanol for lipid mix (mM)	Volume (µL)
Ionizable lipid	50	38.9	5	68.5
Helper lipid	10	10	1	53.3
Cholesterol	39	20	3.9	103.9
C-14 PEG	1	1	0.1	53.3
EtOH				254
Total				533

Table 2

	Priming	Formulation
Volume (mL)	2	1.37
Flow Rate Ratio (Aqueous:EtOH)	3:1	3:1
Total Flow Rate (mL/min)	12	4
Left Syringe Size (mL)	3	3
Right Syringe Size (mL)	1	1
Start Waste Volume (mL)	0.35	0.25
End Waste Volume (mL)	0.05	0.05

Name of Material/ Equipment	Company	Catalog Number
1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (C-14 PEG)	Avanti Polar Lipids	880151P
10 µl Graduated Filter Tips (RNase-,DNase-, DNA-free)	USA Scientific	1121-3810
1000 µl Graduated Filter Tips (RNase-,DNase-, DNA-free)	USA Scientific	1111-2831
20 µl Beveled Filter Tips (RNase-,DNase-, DNA-free)	USA Scientific	1120-1810
200 µl Graudated Filter Tips (RNase-,DNase-, DNA-free)	USA Scientific	1120-8810
3β-Hydroxy-5-cholestene, 5-Cholesten-3β-ol (Cholesterol)	Sigma-Aldrich	C8667
BD Slip Tip Sterile Syringes (1 ml syringe)	Thermo Fisher Scientific	14-823-434
BD Slip Tip Sterile Syringes (3 ml syringe)	Thermo Fisher Scientific	14-823-436
BD Vacutainer General Use Syringe Needles (BD Blunt Fill Needle 18G)	Thermo Fisher Scientific	23-021-020
Benchtop Centrifuge	Beckman coulter	
Black 96 well plates	Thermo Fisher Scientific	14-245-177
BrandTech BRAND BIO-CERT RNase-, DNase-, DNA-free microcentrifuge tubes (1.5mL)	Thermo Fisher Scientific	14-380-813
Citric Acid	Fisher Scientific	02-002-611

Corning 500ml Vacuum Filter/Storage Bottle System, 0.22 um pore	Corning	430769
Disposable folded capillary cells	Malvern	DTS1070
Ethyl Alcohol, Pure 200 proof	Sigma-Aldrich	459844
Fisher Brand Semi-Micro Cuvette	Thermo Fisher Scientific	14955127
Invitrogen Conical Tubes (15 mL) (DNase-RNase-free)	Thermo Fisher Scientific	AM12500
MilliporeSigma Amicon Ultra Centrifugal Filter Units	Thermo Fisher Scientific	UFC901024
NanoAssemblr Benchtop	Precision Nanyosystems	
Nuclease-free water	Thermo Fisher Scientific	AM9930
Phosphate Buffered Saline (PBS)	Thermo Fisher Scientific	AM9624
Quant-iT PicoGreen dsDNA Assay Kit	Thermo Fisher Scientific	P7589
Quant-iT RiboGreen RNA Assay Kit	Thermo Fisher Scientific	R11490
Sodium Chloride	Fisher Scientific	02-004-036
Sodium Citrate, Dihydrate, granular	Fisher Scientific	02-004-056

SpectraMax i3x	Molecular Devices
Zetasizer Nano	Malvern

Comments/Description

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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