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Testing the *In Vitro* and *In Vivo* Efficiency of mRNA-Lipid Nanoparticles Formulated by Microfluidic Mixing

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Abstract

Lipid nanoparticles (LNPs) have attracted widespread attention recently with the successful development of the COVID-19 mRNA vaccines by Moderna and Pfizer/BioNTech. These vaccines have demonstrated the efficacy of mRNA-LNP therapeutics and opened the door for future clinical applications. In mRNA-LNP systems, the LNPs serve as delivery platforms that protect the mRNA cargo from degradation by nucleases and mediate their intracellular delivery. The LNPs are typically composed of four components: an ionizable lipid, a phospholipid, cholesterol, and a lipid-anchored polyethylene glycol (PEG) conjugate (lipid-PEG). Here, LNPs encapsulating mRNA encoding firefly luciferase are formulated by microfluidic mixing of the organic phase containing LNP lipid components and the aqueous phase containing mRNA. These mRNA-LNPs are then tested *in vitro* to evaluate their transfection efficiency in HepG2 cells using a bioluminescent plate-based assay. Additionally, mRNA-LNPs are evaluated *in vivo* in C57BL/6 mice following an intravenous injection *via* the lateral tail vein. Whole-body bioluminescence imaging is performed by using an *in vivo* imaging system. Representative results are shown for the mRNA-LNP characteristics, their transfection efficiency in HepG2 cells, and the total luminescent flux in C57BL/6 mice.

Introduction

Lipid nanoparticles (LNPs) have demonstrated great promise in recent years in the field of non-viral gene therapy. In 2018, the United States Food and Drug Administration (FDA) approved the first-ever RNA interference (RNAi) therapeutic, Onpattro by Alnylam, for the treatment of hereditary transthyretin amyloidosis[1,2,3,4]. This was an important step forward for lipid nanoparticles and RNA-based therapies. More recently, Moderna and Pfizer/BioNTech received FDA approvals for their mRNA-LNP vaccines against SARS-CoV-2[4,5]. In each of these LNP-based nucleic acid therapies, the LNP serves to protect its cargo from degradation by nucleases and facilitate potent intracellular delivery[6,7]. While LNPs have seen success in RNAi therapies and vaccine applications, mRNA-LNPs have also been explored for use in

protein replacement therapies[8] as well as for the co-delivery of Cas9 mRNA and guide RNA for the delivery of the CRISPR-Cas9 system for gene editing[9]. However, there is no one specific formulation that is well-suited for all applications, and subtle changes in the LNP formulation parameters can greatly affect the potency and biodistribution *in vivo*[8,10,11]. Thus, individual mRNA-LNPs must be developed and evaluated to determine the optimal formulation for each LNP-based therapy.

LNPs are commonly formulated with four lipid components: an ionizable lipid, a phospholipid, cholesterol, and a lipid-anchored polyethylene-glycol (PEG) conjugate (lipid-PEG)[11,12,13]. The potent intracellular delivery facilitated by LNPs relies, in part, on the ionizable lipid component[12]. This component is neutral at physiological pH but becomes positively charged in the acidic environment of the endosome[11]. This change in ionic charge is thought to be a key contributor to endosomal escape[12,14,15]. In addition to the ionizable lipid, the phospholipid (helper lipid) component improves the encapsulation of the cargo and aids in endosomal escape, the cholesterol offers stability and enhances membrane fusion, and the lipid-PEG minimizes LNP aggregation and opsonization in circulation[10,11,14,16]. To formulate the LNP, these lipid components are combined in an organic phase, typically ethanol, and mixed with an aqueous phase containing the nucleic acid cargo. The LNP formulation process is very versatile in that it allows for different components to be easily substituted and combined at different molar ratios in order to formulate many LNP formulations with a multitude of physicochemical properties[10,17]. However, when exploring this vast variety of LNPs, it is crucial that each formulation is evaluated using a standardized procedure to accurately measure the differences in characterization and performance.

Here, the complete workflow for the formulation of mRNA-LNPs and the assessment of their performance in cells and animals is outlined.

Protocol

NOTE: Always maintain RNase-free conditions when formulating mRNA-LNPs by wiping the surfaces and equipment with a surface decontaminant for RNases and DNA. Use only RNase-free tips and reagents.

All the animal procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at the University of Pennsylvania and a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

1. Pre-formulation preparation

1. Fill a clean 4 L beaker with 200-300 mL of fresh 10x phosphate-buffered saline (PBS).
    1. Dilute the 10x PBS in the beaker 10-fold with ultrapure water to obtain a beaker of 1x PBS. Ensure that the final volume of the 1x PBS is between 2-3 L.
    2. Place dialysis cassettes (20 kDa molecular weight cutoff [MWCO]) of the appropriate capacity for the LNP formulation into the filled beaker to hydrate them.

        NOTE: Dialysis cassettes require a minimum of 2 min to hydrate before use.
    3. Place a stir bar into the beaker, and cover the beaker with aluminum foil.

4. Place the covered beaker onto a magnetic stir plate. Turn on the stir plate, and set it to spin at 300-400 rpm.

2. Dilute a 100 mM citric acid buffer stock (pH 3) 10-fold with ultrapure water to create the 10 mM citric acid buffer used for mRNA dilution.

3. Make C12-200 ionizable lipid, helper lipid, cholesterol, and lipid-anchored PEG (lipid-PEG) stock solutions by dissolving each lipid in 100% ethanol. The concentrations of the lipid components are detailed in **Table 1**.

    1. Heat and mix the stock solutions at 37 °C to ensure that they are fully dissolved.

2. Preparation of the lipid and nucleic acid mixes

1. Calculate the required volumes of ionizable lipid, helper lipid, cholesterol, and lipid-PEG based on the desired molar ratio and ionizable lipid to mRNA weight ratio (**Table 1**). Prepare 10% extra volume of the organic phase to account for dead volume in the syringes during formulation.

    1. Combine the calculated volumes of the different lipid components in a conical tube.

    2. Dilute the lipids with 100% ethanol to a final volume, *V*, which represents 25% of the final volume of LNP.

2. Calculate the amount of mRNA required for formulation based on the ionizable lipid to mRNA weight ratio and the total volume of mRNA-LNP needed (**Table 1**).

    1. Thaw the firefly luciferase-encoding mRNA on ice.

    2. Dilute the mRNA to a final volume, 3*V*, three times the volume of the organic phase, in 10 mM citric acid buffer.

        NOTE: Every lipid volume, *V*, must contain enough ionizable lipid for the mRNA diluted in 3*V*. This corresponds to the desired ionizable lipid to mRNA weight ratio.

3. Microfluidic formulation of mRNA-LNPs

1. Spray a delicate task wipe with a surface decontaminant for RNases and DNA, and wipe the interior of the microfluidic instrument thoroughly.

    1. Open a new microfluidic cartridge, and insert it with the inlet channels facing down and away.

    2. Ensure that the conical tube arm holds two 15 mL conical tubes and is in the position furthest to the right.

    3. Configure two sterile 15 mL conical tubes on the holder with the caps off.

2. Fill a 5 mL syringe with the mRNA solution that contains mRNA diluted in 10 mM citric acid buffer.

    1. Fill a 3 mL syringe with the lipid solution that contains the lipids diluted in pure 100% ethanol.

    2. Flip up the cartridge holder on the microfluidic device.

    3. Insert the 5 mL syringe, without the needle, that contains mRNA into the left inlet of the cartridge.

    4. Insert the 3 mL syringe, without the needle, that contains lipids into the right inlet of the cartridge.

    5. Flip the cartridge holder back down, and close the lid of the microfluidic instrument.

3. Turn on the microfluidic instrument by using the switch located on the back of the instrument.

    1. Select **Quick Run**.

2. Select the correct syringe types for the 5 mL and 3 mL syringes inserted.
3. Input the parameters for mRNA-LNP formulation at a 3:1 aqueous to organic flow rate ratio as described in **Table 2**.
4. Press the **Next** button to go to the next screen.
5. Confirm that the correct parameters have been inputted.
6. Press the **Start** button to formulate the mRNA-LNPs.

4. Post-formulation processing and characterization of the mRNA-LNPs

1. Load the mRNA-LNPs collected in the right conical tube into the previously hydrated dialysis cassettes.
   NOTE: Do not puncture or damage the membrane of the cassette when loading the mRNA-LNPs. If the membrane is damaged, mRNA-LNPs will be lost upon loading.
   1. Place the dialysis cassettes containing the mRNA-LNPs back into the beaker containing 1x PBS, and leave them to dialyze for a minimum of 2 h.
   2. After dialysis, bring the dialysis cassettes containing the mRNA-LNPs into a sterile biosafety cabinet, and remove the mRNA-LNPs using a syringe with a needle.
   3. Sterile-filter the mRNA-LNPs using a 0.22 µm syringe filter, and collect them in a sterile conical tube.
   4. Place 65 µL of the mRNA-LNP solution into a 1.5 mL microtube for characterization purposes.
2. Dilute 10 µL of mRNA-LNPs 1:100 in 990 µL of 1x PBS in a cuvette for the measurement of the hydrodynamic size and polydispersity index using dynamic light scattering.
3. Assess the concentration and encapsulation efficiency of the mRNA-LNPs using a fluorescence assay (i.e., RiboGreen).
   1. Prepare a stock solution of 1x TE buffer by diluting 20x TE buffer with molecular biology water.
   2. Prepare a stock solution of 1% Triton X-100 buffer by diluting Triton X-100 with 1x TE buffer.
   3. Prepare the fluorescent reagent by diluting the reagent stock 1:200 with 1x TE buffer.
   4. Dilute the mRNA-LNPs 1:100 in 1x TE buffer and 1% Triton X-100 buffer in a black-wall black-bottom 96-well plate in 100 µL.
   5. Prepare a low-range standard curve by diluting the RNA standard as per the manufacturer's instructions, and plate the standard curve in the 96-well plate.
   6. Add 100 µL of diluted fluorescent reagent to each well containing diluted mRNA-LNP or diluted standard.
   7. Place the 96-well plate inside a plate reader, and shake for 1 min, followed by a 4 min wait.
   8. Measure the fluorescence intensity of each well according to the manufacturer's protocol at an excitation/emission of 480 nm/520 nm.
4. Use the standard curve to convert the fluorescence values to concentrations.
   1. Calculate the encapsulation efficiency by subtracting the value obtained from diluting LNPs in TE buffer (unencapsulated mRNA) from the value obtained from diluting LNPs in 1% Triton X-100 (total mRNA) and dividing by the value obtained from diluting LNPs in 1% Triton X-100, according to **equation 4.4**.

2. Calculate the mRNA-LNP concentration used for cell and animal studies by subtracting the value obtained from diluting LNPs in TE buffer (unencapsulated mRNA) from the value obtained from diluting LNPs in 1% Triton X-100 (total mRNA).

5. Measure the pK_a of the mRNA-LNPs by performing a 6-(p-toluidino)-2-naphthalenesulfonyl chloride (TNS) assay.
   1. Prepare the TNS reagent by creating a 0.16 mM solution of TNS in ultrapure water.
      NOTE: When using the reagent, be sure to keep it on ice and away from light to avoid degradation.
   2. Prepare buffered solutions of 150 mM sodium chloride, 20 mM sodium phosphate, 25 mM ammonium citrate, and 20 mM ammonium acetate adjusted to different pH values ranging from 2 to 12 in increments of 0.5.
   3. Add 2.5 µL of the LNP to each pH-adjusted buffer solution in a black-wall black-bottom 96-well plate.
   4. Add TNS reagent to each well so that the final TNS concentration is 6 µM.
   5. Incubate the 96-well plate in a dark location for 5 min.
   6. Measure the fluorescence at an excitation/emission of 322 nm/431 nm using a fluorescence plate reader.
   7. Fit the data to a sigmoidal curve, and calculate the pK_a by using the fitted equation to find the pH at which 50% of the maximum intensity was observed.

6. Representative results for the mRNA-LNP hydrodynamic size, PDI, encapsulation efficiency, and pK_a are shown in **Table 3**.

5. *In vitro* transfection of HepG2 cells

NOTE: Various other cell lines, such as HeLa cells or HEK-293T cells, may be used for assessing the transfection efficiency of LNPs *in vitro*. All cells should test negative for mycoplasma prior to the LNP transfection studies.

1. Plate 5,000 HepG2 cells in each well of a white-wall clear-bottom 96-well plate in 100 µL of complete cell culture medium (DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin)
   1. Leave the cells to adhere overnight in a controlled CO_2 incubator at 37 °C and 5% CO_2.
2. Dilute the mRNA-LNPs in complete cell culture medium so that the total dose is delivered in 100 µL.
   1. Remove the complete medium from the wells.
   2. Treat the cells with either mRNA-LNPs diluted in complete cell culture medium at the desired doses or complete medium (control).
   3. Place the 96-well plate containing the treated cells back into the controlled CO_2 incubator for 24 hours.
3. Assess the transfection efficiency by performing a luciferase assay.
   1. Prepare the luciferase reagent and 1x cell lysis buffer as per the manufacturer's instructions.
   2. Take out the 96-well plate containing the cells from the incubator, and bring it into a biosafety cabinet.
   3. Remove the cell culture medium from every well of the 96-well plate.
   4. Add 20 µL of 1x lysis buffer to each well, followed by 100 µL of the previously prepared luciferase assay reagent.

5. Protect the plate from light, and place the plate into a plate reader to measure the bioluminescent signal of each well.

   NOTE: Representative results demonstrating the treatment of HepG2 cells with previously formulated C12-200 mRNA-LNPs are shown in **Figure 1**.

6. *In vivo* evaluation of mRNA-LNPs in mice following tail vein injection

1. Dilute the mRNA-LNP solution with sterile 1x PBS so that 2 µg of total mRNA is present in a 100 µL tail vein injection volume, which corresponds to a body weight dose of approximately 0.1 mg/kg for a 20 g mouse.
2. Restrain each mouse using an approved method, and wipe the tail with a pad moistened with 70% alcohol.
   1. Fill a 29 G insulin syringe with either 100 µL of mRNA-LNP (2 µg) or 100 µL of 1x PBS. Remove any bubbles from the syringe.
   2. Insert the needle with the bevel facing up into the mouse's lateral tail vein, and slowly inject the 100 µL of mRNA-LNP or 1x PBS.
   3. Remove the needle and apply pressure until hemostasis is achieved.
3. Prepare a stock solution of 15 mg/mL d-luciferin potassium salt in sterile 1x PBS 6 hours post injection.
   1. Turn on the *in vivo* imaging system (IVIS), and open the imaging software.
   2. Press **Initialize** to prepare the instrument for data acquisition.
   3. Check the box next to luminescence, and set the exposure time to auto. Ensure that the correct field of view is selected for the number of mice being imaged.
4. Administer 200 µL of d-luciferin intraperitoneally (150 mg of luciferin per kg body weight) to the previously treated mice.
5. Place the mice into an anesthesia chamber set to 2.5% isoflurane and an oxygen flowrate of 2.0 L/min.
6. Wait 10 min for the luciferase signal to stabilize before imaging the mRNA-LNP-treated mice.
7. Ensure that the mice are fully anesthetized, and redirect the anesthetic line to administer isoflurane *via* nose cones inside the imaging chamber.
8. Transfer the mice to the nose cones, and ensure that they are on their backs with their abdomens exposed.
9. Close the chamber door, and click the **Acquire** button in the software to obtain bioluminescence images.

   NOTE: Representative results for mouse whole-body imaging following mRNA-LNP or 1X PBS treatment are shown in **Figure 2**.

Representative Results

mRNA-LNPs were formulated using a microfluidic instrument that possessed an average hydrodynamic diameter of 76.16 nm and a polydispersity index of 0.098. The pK_a of the mRNA-LNPs was found to be 5.75 by performing a TNS assay[18]. The encapsulation efficiency for these mRNA-LNPs was calculated to be 92.3% by using the modified fluorescence assay and **equation 4.4**. The overall RNA concentration that was used for the cell treatment and animal dosing was 40.24 ng/µL. This value was obtained from the modified fluorescence assay[19], specifically from converting

the fluorescence obtained by diluting the LNPs with 1% Triton X-100 and 1x TE buffer to certain concentrations and calculating the amount of encapsulated mRNA.

Equation 4.4

$$EE\% = \frac{(C_{TX}) - (C_{TE})}{(C_{TX})} \times 100$$

C_{TX} = Concentration of mRNA from LNPs diluted in 1% Triton X-100

C_{TE} = Concentration of mRNA from LNPs diluted in 1x TE buffer

Increasing bioluminescence was observed with increasing doses upon the treatment of 5,000 HepG2 cells per well with different doses of mRNA-LNP. Luciferase expression was easily detected at the lowest dose (5 ng/well) used in this study. However, other cell lines may require different amounts of mRNA-LNP to readily see differences in luminescence across doses.

Mice were treated with 2 µg of mRNA-LNP and assessed 6 h later for whole-body bioluminescence. As C12-200 mRNA-LNPs predominantly transfect the liver[20], a strong bioluminescent signal was observed in the upper abdomen of the mice treated with our formulated LNPs. Using the imaging software, regions of interest were drawn around the liver signals to calculate the total luminescent flux. In this experiment, the total luminescent flux was approximately 5 x 10^9, which is consistent with other studies conducted with this LNP formulation[4,21].

Table 1: Organic phase composition. Concentrations of the individual lipid stock solutions and the volumes that each component contributes to the 1.3 mL organic phase are described. Lipids are combined at a molar ratio of 35/16/46.5/2.5 (C12-200:DOPE:Cholesterol:C14-PEG2000). A 10% extra volume was prepared to account for syringe dead volumes during formulation. Please click here to download this Table.

Table 2: Microfluidic instrument settings for mRNA-LNP formulation at a 3:1 aqueous to organic flow rate ratio. The 5 mL syringe containing mRNA diluted in 10 mM citric acid buffer was inserted into the center channel, while the 3 mL syringe containing lipids was inserted into the right channel. LNPs were formulated at a 10:1 weight ratio of ionizable lipid:mRNA. Please click here to download this Table.

Table 3: mRNA-LNP characterization data determined by dynamic light scattering, a modified fluorescenceassay, and a 2-(p-toluidino) naphthalene-6-sulfonic acid (TNS) assay. Please click here to download this Table.

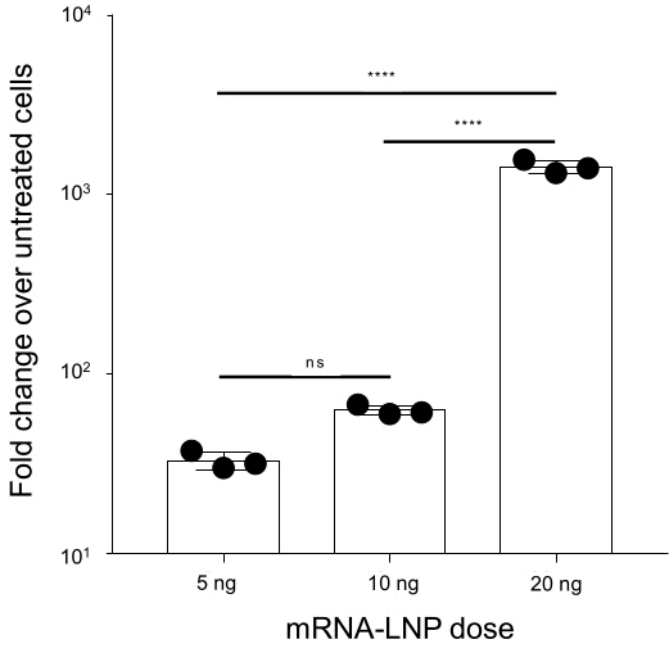

Figure 1: HepG2 cells treated with firefly luciferase mRNA-LNP. Overall, 5,000 HepG2 cells were seeded per well in a 96-well plate and allowed to adhere overnight. The cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cell culture medium was removed before cells were treated with 5 ng, 10 ng, and 20 ng doses of a C12-200-containing LNP formulation encapsulating firefly luciferase-encoding mRNA (in 100 µL of cell culture medium). At 24 h post treatment, the cell culture medium was removed, and 20 µL of 1x lysis buffer was added onto the cells before the addition of 100 µL of luciferase assay reagent. The bioluminescence from each well was measured using a plate reader following a 5 min incubation period. The fold increases are plotted relative to control wells that consisted of untreated HepG2 cells. The statistical significance was evaluated using a one-way ANOVA with post-hoc Tukey's test. ns, not significant; ****, $p < 0.0001$. Please click here to view a larger version of this figure.

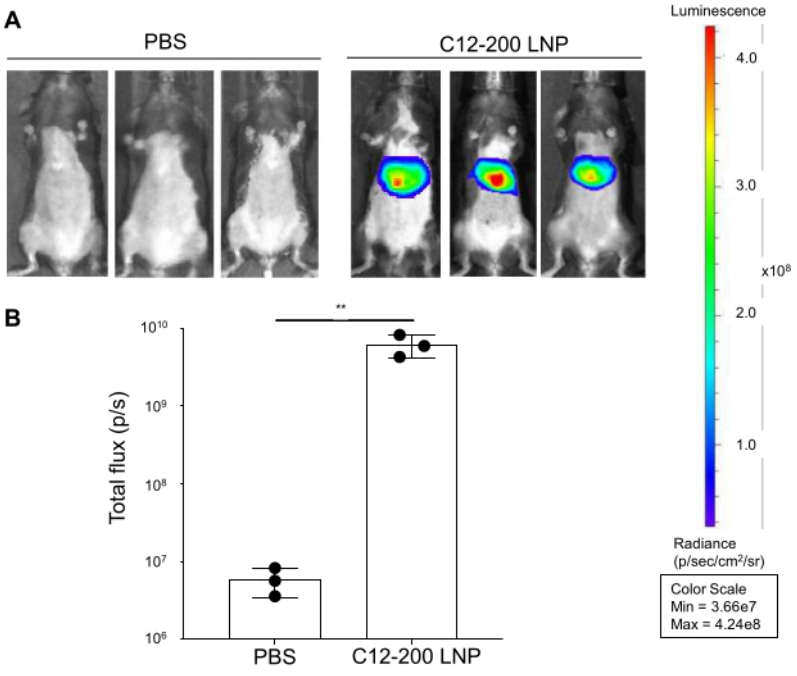

Figure 2: C57BL/6 mice treated with firefly luciferase mRNA-LNP. In this work, 2 µg of total mRNA-LNP in 100 µL or 100 µL of 1x PBS was administered to C57BL/6 mice *via* the lateral tail vein. (**A**) Whole-body bioluminescence of the mice treated with either mRNA-LNP or 1X PBS was measured using the *in vivo* imaging system (IVIS) 6 h post administration. The IVIS Spectrum Living Image software was used to analyze and acquire the whole-body images. N = 3. (**B**) The total luminescent flux was quantified and plotted. The statistical significance was evaluated using a two-tailed Student's t-test. **, $p < 0.01$. Please click here to view a larger version of this figure.

Discussion

With this workflow, a variety of mRNA-LNPs can be formulated and tested for their *in vitro* and *in vivo* efficiency. Ionizable lipids and excipients can be swapped out and combined at different molar ratios and different ionizable lipid to mRNA weight ratios to produce mRNA-LNPs with differing physicochemical properties[22]. Here, we formulated C12-200 mRNA-LNPs with a molar ratio of 35/16/46.5/2.5 (ionizable lipid:helper lipid:cholesterol:lipid-PEG) at a 10:1 ionizable lipid to mRNA weight ratio. These LNPs were tested for their transfection efficiency *in vitro* in HepG2 cells and *in vivo* in C57BL/6 mice. The C12-200 mRNA-LNPs were synthesized by microfluidic mixing using a commercially available microfluidic instrument. Though other formulation methods can be used to formulate LNPs, such as pipette mixing or T-junction mixing[4], microfluidic mixing formulates mRNA-LNPs that are generally smaller, less polydisperse, and have higher encapsulation efficiencies[4,13,23,24].

The formulation of mRNA-LNPs with the commercially available microfluidic instrument allows for the simple and flexible formulation of LNPs. The flow rate at the beginning of a run varies from the target flow rate set as the syringe

pushers accelerate to full speed. This can lead to variable particle characteristics and inconsistent results. To overcome this, the microfluidic instrument was designed to collect the LNPs formulated at the start and end of a formulation as waste separately from the LNPs formulated at steady-state conditions. This can decrease the polydispersity and lead to more consistent particle properties. The start waste can be calculated based on the combination of syringes being used for formulation as per the manufacturer's manual. However, the end waste does not experience the same degree of non-steady-state flow conditions, so the volume is often set to 0.05 mL for all syringe combinations as per the manufacturer's recommendation.

It is critical to account for dead volume in syringes and tubes when preparing the correct volumes of the organic and aqueous phases. Preparing 10% extra volume of each phase ensures that the minimum volume of LNP desired can be formulated. In this protocol, a total formulation volume of 4.7 mL was inputted with 0.7 mL of waste, resulting in 4 mL of LNP collected in steady-state conditions. However, 1.3 mL of the organic phase and 3.9 mL of the aqueous phase were prepared for formulation. This ensures that the 5 mL syringe can be filled up to the 3.6 mL mark and the 3 mL syringe can be filled up to the 1.2 mL mark. If the volume reaches the marks on the syringes, then the volume specifications used on the microfluidic instrument are safe inputs. The final screen before pressing the "START" button is crucial for ensuring that all the parameters are correct. It is important to do a final check on the syringe types, the volumes in the syringes, the volumes to be dispensed, the syringe placement, and the flow rates.

After the formulation of mRNA-LNPs, the dialysis step has a few important considerations to keep in mind. The dialysis cassettes used require a minimum hydration time of 2 min before the LNPs can be loaded. This increases the membrane flexibility and allows the membrane to adjust more readily as the sample is added. Additionally, care must be taken not to damage or puncture the membranes of the dialysis cassettes while loading them with mRNA-LNPs. If the membranes become punctured or damaged, the formulated mRNA-LNPs can be easily lost upon loading.

In this protocol, we tested the *in vitro* efficiency of mRNA-LNPs in HepG2 cells. However, other cell types can be tested for mRNA-LNP transfection efficiency. If non-adherent cells are being used, the 96-well plate requires centrifugation at 500 *g* for 5 min prior to any removal of the cell culture medium[25]. This minimizes any loss of cells during the medium removal. The cells should also be tested for the presence of mycoplasma prior to any LNP transfection studies. It will be difficult to obtain consistent results and compare different LNP formulations if the cells test positive for mycoplasma. Additionally, primary cells may require higher doses of LNP to measure transfection without toxicity compared with cell lines. A luciferase-based viability assay can be used to evaluate the mRNA-LNP toxicity *in vitro* at varying doses in both primary cells and cell lines[18].

One of the most important steps in obtaining consistent bioluminescence measurements across different mice following treatment with the same mRNA-LNP formulation is ensuring that the time between the intraperitoneal injection of d-luciferin and the IVIS measurement is consistent[26]. This time interval influences the stability of the bioluminescent signal obtained. The time interval needs to be long enough so that any fluctuations in the signal are minimized. A preliminary experiment can be run in which the mice are imaged every minute following the d-luciferin injection. The time interval

when the signal starts to plateau is the one most suitable for the transfection experiment. In this protocol, a 10 min interval allowed for stable luminescence signals following the 150 mg d-luciferin/kg body weight injection, which is commonly performed for imaging[27,28].

Additionally, the mRNA-LNP dose used in this protocol (0.1 mg mRNA/kg body weight) is small compared to the doses used to assess toxicity and therapeutic efficacy[29,30]. These smaller doses help to evaluate differences in the potencies of unique mRNA-LNP formulations while not oversaturating the luminescent signal obtained. However, this dose can be increased to measure the potential mRNA-LNP toxicity. For example, liver toxicity can be evaluated by quantifying the levels of alanine transaminase, aspartate transaminase, and alkaline phosphatase in the serum at different time points following mRNA-LNP injection[31,32,33]. These enzymes are normally found at low levels in the serum but are increased as a result of liver damage. Commercially available kits can be used to quantify the amount of these enzymes in the serum.

In this example, we used firefly luciferase-encoding mRNA, but other reporter mRNAs can be formulated into LNPs to assess potency. mRNAs encoding for green fluorescent protein (GFP) or mCherry can be employed to investigate cell-specific delivery following mRNA-LNP treatment[34,35]. A single-cell suspension can be produced from tissues of interest, and GFP^+ or $mCherry^+$ cells can be assessed by flow cytometry analysis. Additionally, mRNA-LNPs can be formulated with mRNA encoding for erythropoietin in order to measure liver transfection through the secretion of erythropoietin in the serum[8,36,37]. In Ai9 mice, the delivery of mRNA encoding Cre recombinase induces robust tdTomato fluorescence, which can serve as another method for investigating mRNA-LNP delivery to different cell populations[38,39,40]. Through the demonstration of this workflow, it is our hope that mRNA-LNP formulation ceases to be a barrier for investigators as they explore new ideas and possibilities for mRNA therapeutics.

Disclosures

There are no conflicts of interest to declare.

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References

1. Cheng, Q. et al. Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR-Cas gene editing. *Nature Nanotechnology.* **15** (4), 313-320 (2020).
2. Wood, H. FDA approves patisiran to treat hereditary transthyretin amyloidosis. *Nature Reviews Neurology.* **14** (9), 509 (2018).
3. Zhang, X., Goel, V., Robbie, G. J. Pharmacokinetics of patisiran, the first approved RNA interference therapy in patients With hereditary transthyretin-mediated amyloidosis. *Journal of Clinical Pharmacology.* **60** (5), 573-585 (2019).
4. Shepherd, S. J. et al. Scalable mRNA and siRNA lipid nanoparticle production using a parallelized microfluidic device. *Nano Letters.* **21** (13), 5671-5680 (2021).

5. Barbier, A. J., Jiang, A. Y., Zhang, P., Wooster, R., Anderson, D. G. The clinical progress of mRNA vaccines and immunotherapies. *Nature Biotechnology.* **40** (6), 840-854 (2022).
6. Mukalel, A. J., Riley, R. S., Zhang, R., Mitchell, M. J. Nanoparticles for nucleic acid delivery: Applications in cancer immunotherapy. *Cancer Letters.* **458**, 102-112 (2019).
7. Akhtar, S. Oral delivery of siRNA and antisense oligonucleotides. *Journal of Drug Targeting.* **17** (7), 491-495 (2009).
8. Guimaraes, P. P. G. et al. Ionizable lipid nanoparticles encapsulating barcoded mRNA for accelerated in vivo delivery screening. *Journal of Controlled Release.* **316**, 404-417 (2019).
9. Qiu, M. et al. Lipid nanoparticle-mediated codelivery of Cas9 mRNA and single-guide RNA achieves liver-specific in vivo genome editing of Angptl3. *Proceedings of the National Academy of Sciences of the United States of America.* **118** (10), e2020401118 (2021).
10. Zhang, R. et al. Helper lipid structure influences protein adsorption and delivery of lipid nanoparticles to spleen and liver. *Biomaterials Science.* **9** (4), 1449-1463 (2021).
11. El-Mayta, R. et al. A nanoparticle platform for accelerated in vivo oral delivery screening of nucleic acids. *Advanced Therapeutics.* **4** (1), 2000111 (2021).
12. Patel, S. et al. Naturally-occurring cholesterol analogues in lipid nanoparticles induce polymorphic shape and enhance intracellular delivery of mRNA. *Nature Communications.* **11**, 983 (2020).
13. Kulkarni, J. A. et al. Design of lipid nanoparticles for in vitro and in vivo delivery of plasmid DNA. *Nanomedicine: Nanotechnology, Biology, and Medicine.* **13** (4), 1377-1387 (2017).
14. Cheng, X., Lee, R. J. The role of helper lipids in lipid nanoparticles (LNPs) designed for oligonucleotide delivery. *Advanced Drug Delivery Reviews.* **99** (Pt A), 129-137 (2016).
15. Varkouhi, A. K., Scholte, M., Storm, G., Haisma, H. J. Endosomal escape pathways for delivery of biologicals. *Journal of Controlled Release.* **151** (3), 220-228 (2011).
16. Granot, Y., Peer, D. Delivering the right message: Challenges and opportunities in lipid nanoparticles-mediated modified mRNA therapeutics-An innate immune system standpoint. *Seminars in Immunology.* **34**, 68-77 (2017).
17. Gan, Z. et al. Nanoparticles containing constrained phospholipids deliver mRNA to liver immune cells in vivo without targeting ligands. *Bioengineering and Translational Medicine.* **5** (3), e10161 (2020).
18. Patel, S. K. et al. Hydroxycholesterol substitution in ionizable lipid nanoparticles for mRNA delivery to T cells. *Journal of Controlled Release.* **347**, 521-532 (2022).
19. Robinson, E. et al. Lipid nanoparticle-delivered chemically modified mRNA restores chloride secretion in cystic fibrosis. *Molecular Therapy.* **26** (8), 2034-2046 (2018).
20. Love, K. T. et al. Lipid-like materials for low-dose, in vivo gene silencing. *Proceedings of the National Academy of Sciences of the United States of America.* **107** (5), 1864-1869 (2010).
21. Kauffman, K. J. et al. Optimization of lipid nanoparticle formulations for mRNA delivery in vivo with fractional

factorial and definitive screening designs. *Nano Letters.* **15** (11), 7300-7306 (2015).

22. Billingsley, M. M. et al. Ionizable lipid nanoparticle-mediated mRNA delivery for human CAR T cell engineering. *Nano Letters.* **20** (3), 1578-1589 (2020).
23. Ramaswamy, S. et al. Systemic delivery of factor IX messenger RNA for protein replacement therapy. *Proceedings of the National Academy of Sciences of the United States of America.* **114** (10), E1941-E1950 (2017).
24. Leung, A. K. K. et al. Lipid nanoparticles containing siRNA synthesized by microfluidic mixing exhibit an electron-dense nanostructured core. *Journal of Physical Chemistry C.* **116** (34), 18440-18450 (2012).
25. Billingsley, M. M. et al. Orthogonal design of experiments for optimization of lipid nanoparticles for mRNA engineering of CAR T cells. *Nano Letters.* **22** (1), 533-542 (2022).
26. Khalil, A. A. et al. Subcutaneous administration of D-luciferin is an effective alternative to intraperitoneal injection in bioluminescence imaging of xenograft tumors in nude mice. *ISRN Molecular Imaging.* **2013**, 689279 (2013).
27. Qin, J. et al. RGD peptide-based lipids for targeted mRNA delivery and gene editing applications. *RSC Advances.* **12** (39), 25397-25404 (2022).
28. Pardi, N. et al. Expression kinetics of nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes. *Journal of Controlled Release.* **217**, 345-351 (2015).
29. Finn, J. D. et al. A single administration of CRISPR/Cas9 lipid nanoparticles achieves robust and persistent in vivo genome editing. *Cell Reports.* **22** (9), 2227-2235 (2018).
30. Truong, B. et al. Lipid nanoparticle-targeted mRNA therapy as a treatment for the inherited metabolic liver disorder arginase deficiency. *Proceedings of the National Academy of Sciences of the United States of America.* **116** (42), 21150-21159 (2019).
31. Cheng, Q. et al. Dendrimer-based lipid nanoparticles deliver therapeutic FAH mRNA to normalize liver function and extend survival in a mouse model of hepatorenal tyrosinemia type I. *Advanced Materials.* **30** (52), e1805308 (2018).
32. Sedic, M. et al. Safety evaluation of lipid nanoparticle-formulated modified mRNA in the Sprague-Dawley rat and cynomolgus monkey. *Veterinary Pathology.* **55** (2), 341-354 (2018).
33. Veiga, N. et al. Cell specific delivery of modified mRNA expressing therapeutic proteins to leukocytes. *Nature Communications.* **9** (1), 4493 (2018).
34. Pattipeiluhu, R. et al. Anionic lipid nanoparticles preferentially deliver mRNA to the hepatic reticuloendothelial system. *Advanced Materials.* **34** (16), e2201095 (2022).
35. Rosenblum, D. et al. CRISPR-Cas9 genome editing using targeted lipid nanoparticles for cancer therapy. *Science Advances.* **6** (47), eabc9450 (2020).
36. Fenton, O. S. et al. Bioinspired alkenyl amino alcohol ionizable lipid materials for highly potent in vivo mRNA delivery. *Advanced Materials.* **28** (15), 2939-2943 (2016).
37. Kauffman, K. J. et al. Optimization of lipid nanoparticle formulations for mRNA delivery in vivo with fractional

factorial and definitive screening designs. *Nano Letters.* **15** (11), 7300-7306 (2015).

38. Tombácz, I. et al. Highly efficient CD4+ T cell targeting and genetic recombination using engineered CD4+ cell-homing mRNA-LNPs. *Molecular Therapy.* **29** (11), 3293-3304 (2021).
39. Kim, J. et al. Engineering lipid nanoparticles for enhanced intracellular delivery of mRNA through inhalation. *ACS Nano.* **16** (9), 14792-14806 (2022).
40. Bevers, S. et al. mRNA-LNP vaccines tuned for systemic immunization induce strong antitumor immunity by engaging splenic immune cells. *Molecular Therapy.* **30** (9), 3078-3094 (2022).