بسم الله الرحمن الرحيم

MEGBI - Middle East Genetics and Biotechnology Institute
مركز أبحاث للجينات والتقنية البيولوجية
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AECENAR TECDA
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MEGBI-Covid19 Vaccine Production

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1 Introduction

As the world continues to fight the COVID-19 pandemic and prepares for the global distribution of vaccines, the global need for vaccine and the wide geographic diversity of the pandemic requires more than one effective vaccine approach.. The full development pathway for an effective vaccine for SARS-CoV-2 will require that industry, government, and academia collaborate in unprecedented ways, each adding their individual strengths. In this report we have acquired some protocols that can be generalized for the production of RNA vaccine.

2 Needed devices

2.1 Devices already in MEGBI Lab

2.2 Homoginizer:

Note: Please be cautious and check with your supplier if this product is for virus protection purposes and if the coronavirus (COVID-19) will affect your order.

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Laboratory Tissue High Shear Mixer Homogenizer with Digital Display

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$461.00 / Piece | 1 Piece (Min. Order)

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1 - 2 Sets	3 - 4 Sets	>=5 Sets
$1,000.00	**$990.00**	**$980.00**

Model Number: Model:KQFS-300N

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Shipping: Support Express · Sea freight · Air freight

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3 Needed material

- **FROM PROMEGA:**
  - REVERSE TRANSCRIPTION SYSTEM: PRODUCT A3500 368.92 USD
  - RiboMAX Large Scale RNA Production System-T7: PRODUCT P1300 531.96 USD
- **FROM THERMOFISHER**
  - Dynabeads™ mRNA Purification Kit: CATALOG NUMBER: 61006 price N/A
    http://www.molecularcloning.com/index.php?prt=110

4 Reverse transcription protocol (Reverse Transcription System INSTRUCTIONS FOR USE OF PRODUCT A3500.)

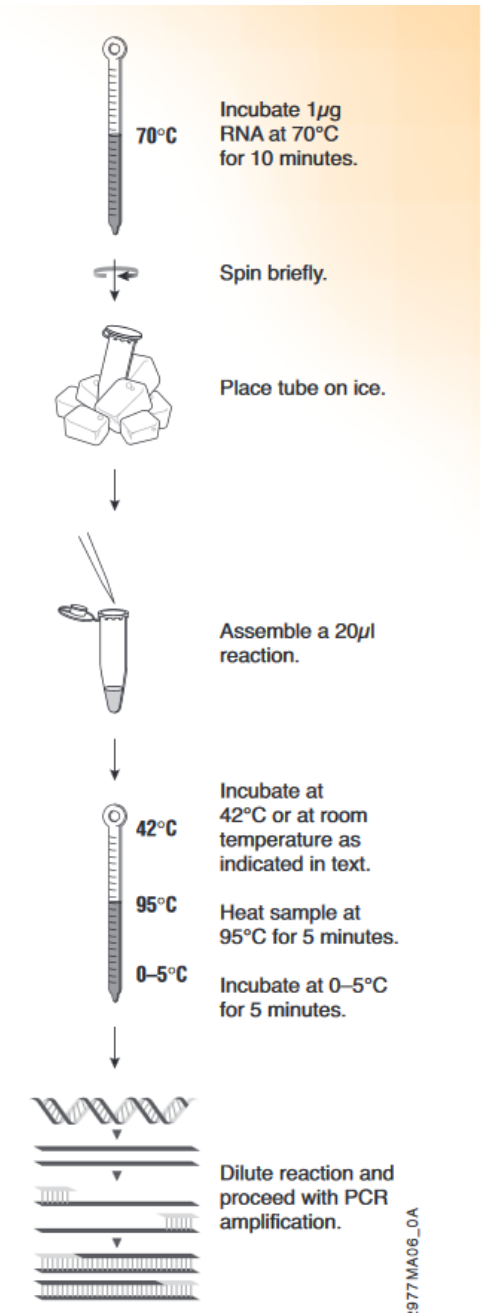
4.1 Reverse Transcription Reaction (First-Strand cDNA Synthesis)

1. Place 1μg (2μl) of 1.2kb Kanamycin Positive Control RNA, poly(A)+ mRNA ortotal RNA in a microcentrifuge tube, and incubate at 70°C for 10 minutes. Centrifuge briefly in a microcentrifuge, then place on ice.

2. Prepare a 20μl reaction by adding the following reagents in the order listed(this reaction can be scaled up or down, depending on the amount of RNA).

3. When using Oligo(dT)15Primer, incubate the reaction at 42°C for 15 minutes. When using Random Primers, incubate the reaction at room temperature for10 minutes, then incubate at 42°C for 15 minutes.

4. Heat the sample at 95°C for 5 minutes, then incubate at 0–5°C for 5 minutes.



4.2 Dilution of the Reaction for Amplification

1. Dilute the first-strand cDNA synthesis reaction to 100µl with TE buffer or Nuclease-Free Water.

2. Prepare a 100µl PCR amplification mix by combining the following reagents.Template-specific upstream and downstream primers should be used for this reaction.

Component	Amount
first-strand cDNA reaction	10–20µl
dNTP Mixture, 10mM	1.8µl
$MgCl_2$, 25mM	7.5µl
Reverse Transcription 10X Buffer	9.8µl
upstream primer	50pmol
downstream primer	50pmol
Taq DNA polymerase	2.5 units
Nuclease-Free Water to a final volume of	100µl

3. Proceed to thermal cycling according to your own specific experiment.

5 Transcription Protocol

5.1 Before You Begin

- Prepare solution of chloroform:isoamyl alcohol (24:1)
- Prepare TE-saturated (pH 8.0) phenol:chloroform:isoamyl alcohol (25:24:1)
- Prepare solutions of 70% and 95% ethanol
- Prepare RNA loading buffer
- Prepare RNA sample buffer
- Prepare MOPS buffer
- Prepare 25mM rNTPs

5.2 Preparation and Linearization of Template DNA

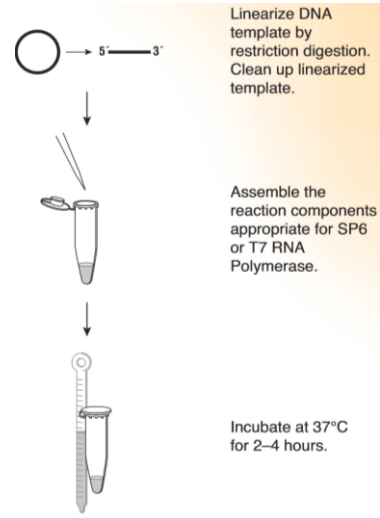
Templates should be linearized by digestion with the appropriate restriction endonuclease followed by a cleanup procedure, such as the Wizard® DNA Clean-Up System or phenol extraction followed by ethanol precipitation.

5.3 Transcription Procedure

1. Assemble the reaction components appropriate for SP6 or T7 RNA Polymerase at room temperature in a 1.5ml microcentrifuge tube. After all the components are added, mix by pipetting gently.

SP6 Reaction Components	Sample Reaction
SP6 Transcription 5X Buffer	20µl
rNTPs (25mM ATP, CTP, GTP, UTP)	20µl
linear DNA template (5–10µg total)	
plus Nuclease-Free Water	50µl
Enzyme Mix (SP6)	10µl
final volume	100µl
T7 Reaction Components	**Sample Reaction**
T7 Transcription 5X Buffer	20µl
rNTPs (25mM ATP, CTP, GTP, UTP)	30µl
linear DNA template (5–10µg total)	
plus Nuclease-Free Water	40µl
Enzyme Mix (T7)	10µl
final volume	100µl

Larger scale reactions may be performed by increasing ALL volumes proportionally.



2. Pipet gently and incubate at 37°C for 2–4 hours. DNA templates can be removed by DNase treatment. RNA can be visualized by gel electrophoresis.

6 Purification protocol 1:

Thermofisher scientific catalog number 61006

The following protocol describes mRNA isolation using 75 µg of total RNA as starting material. This protocol can be scaled up or down by increasing or decreasing reagent volumes proportionally with any changes in the amount of the total RNA starting material. Optimization may be needed.

6.1 Prepare rna:

1. Adjust the volume of the total RNA sample (75 µg) to 100 µL with distilled DEPC-treated water, or with 10 mM Tris-HCl, pH 7.5. Omit this step if only a small adjustment is needed (see step 3 of "Prepare Dynabeads™ magnetic beads").

2. Heat the sample to 65°C for 2 minutes to disrupt secondary structures.

3. Place sample on ice.

Prepare Dynabeads™ magnetic beads

1. Transfer 200 µL (1 mg) of resuspended Dynabeads™ magnetic beads to a microcentrifuge tube. Place the tube on the magnet for 30 seconds, or until all the Dynabeads™ magnetic beads have adhered to the tube wall.

2. Discard the supernatant, remove the tube from the magnet, and add 100 µL of Binding Buffer to equilibriate the beads. Place the tube on the magnet and remove the supernatant. Remove the tube from the magnet.

3. Add 100 µL Binding Buffer to the Dynabeads™ magnetic beads. Optimal hybridization conditions require a 1:1 ratio of Binding Buffer to sample volume. If the total RNA is more dilute than 75 µg/100 µL, then add a volume of Binding Buffer equal to the sample volume to the Dynabeads™ magnetic beads.

6.2 Isolate mRNA

1. Add the total RNA to the Dynabeads™/Binding Buffer suspension. Mix thoroughly, and rotate on a roller or mixer for 3–5 minutes at room temperature to allow the mRNA to anneal to the oligo (dT)25 on the beads.

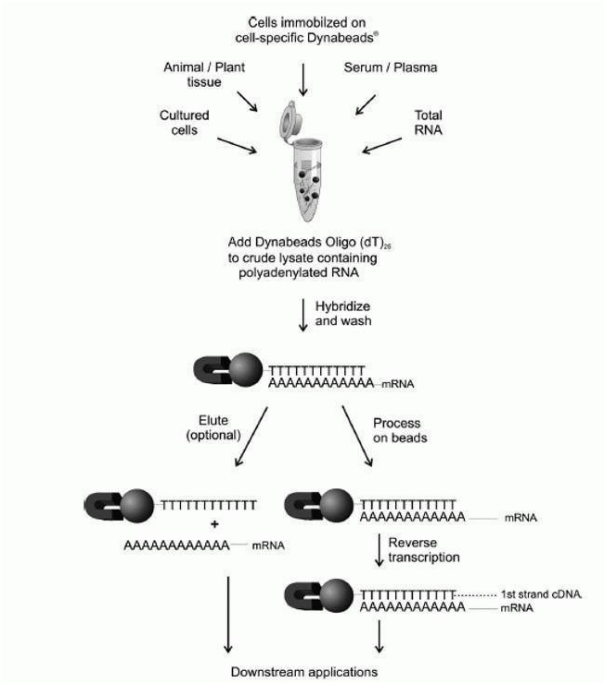
2. Place the tube on the magnet until the solution is clear. Remove the supernatant.

3. Remove the tube from the magnet and wash the mRNA-bead complex twice with 200 µL Washing Buffer B. Use the magnet to remove all traces of supernatant between each washing step (this is important when working with small volumes).

4. (Optional) Add 10–20 µL (or down to 5 µL) of 10 mM Tris-HCl, pH 7.5 to elute the mRNA.

5. Heat the sample at 65°C to 80°C for 2 minutes and place the tube immediately on the magnet.

6. Transfer the eluted mRNA to a new RNase-free tube.



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7 Purification protocol 2: Absolutely mRNA Purification Kit Catalog #400806

PROTOCOL

The Absolutely mRNA purification protocol given below is for purification of mRNA from 100 µg total RNA. Reactions may be scaled down to 0.5× (for 50 µg total RNA) or up to 10× (for up to 1 mg total RNA) in a single reaction tube. When scaling up or down, simply increase or decrease the volumes of all components, including the Absolutely mRNA oligo (dT) magnetic particles.

1. Prepare the total RNA (100 µg) in 100 µl of Absolutely mRNA elution buffer or 100 µl of RNase-free H2O.

Note If the total RNA is more dilute than 1 µg/µl, the 100 µg of RNA may be added in a larger sample volume. In this case, increase the volume of Absolutely mRNA hybridization buffer used in step 4 to equal the initial volume of the total RNA sample.

2. Heat the total RNA solution to 65°C for 3 minutes, and then transfer the sample to ice.

3. Swirl or roll the vial of Absolutely mRNA oligo (dT) magnetic particles to ensure that the particles are in a homogeneous suspension. Transfer 50 µl of the particle suspension to an RNase-free microcentrifuge tube. Wash the particles twice with 100 µl of Absolutely mRNA hybridization buffer. During each wash, mix by pipetting and then collect the beads using a magnetic separator stand, according to the manufacturer's instructions.

Notes The hybridization buffer may precipitate when stored at cooler temperatures. Store the buffer at room temperature and do not place the buffer on ice. If a precipitate is present, warm the buffer to 50°C and swirl the solution to redissolve the components.

Whenever the particles are collected using the magnetic separator, leave the tube in the magnetic stand while removing the supernatant.

4. Resuspend the washed particles in 100 µl of Absolutely mRNA hybridization buffer by repeated pipetting.

5. Add 100 µl of the total RNA solution to the resuspended magnetic particles. Mix by repeated pipetting.

6. Incubate the mixture at room temperature with gentle agitation for 5 minutes to allow hybridization of the polyA-RNA to the particles.

7. Collect the magnetic particles complexed with mRNA using a magnetic separator stand.

4 Absolutely mRNA Purification Kit

8. Remove the supernatant with a pipet tip.

Note The supernatant, containing the unbound fraction of the total RNA may be retained, if desired, to facilitate troubleshooting or may be discarded at this step.

9. Wash the mRNA-bound magnetic particles four times using 100 µl of Absolutely mRNA wash buffer for each wash. Gently resuspend the particles during each wash, and then re-collect the particles using the magnetic separator stand. Be sure to remove all of the wash buffer when completing the final wash.

10. Remove the tube from the magnetic stand and then add 100 µl of Absolutely mRNA elution buffer to the particles. Incubate the tube at room temperature with gentle agitation for 5 minutes to release the mRNA from the particles.

11. Collect the magnetic particles using a magnetic separator stand.

12. Draw off the 100-µl eluate, containing the purified mRNA, and transfer the solution to a fresh, RNase-free tube. The RNA can be stored at –20°C for up to one month or at –80°C for long-term storage.

7.1 Expected RNA Yields and Quantification

To quantify mRNA isolated from 50 µg–1 mg total RNA, a highly sensitive fluorescence-based system (e.g., RiboGreen® RNA quantitation kit, Molecular Probes, Inc.) may be used. The expected yield of mRNA is 1–5% of the amount of total RNA starting material

7.2 mRNA characterization

mRNA samples were characterized using the E-Gel iBase PowerSystem with E-Gel EX gels (ThermoFisher) under denaturing con-ditions with 90% formamide. Gels were imaged using a BioRadChemiDoc MP imager. Size fractionation was performed with anAgilent 2100 BioAnalyzer (Santa Clara,

CA) at an mRNA concen-tration of 0.6mg/mL. An RNA ladder (200, 500, 1000, 2000, 4000,6000 nt) was used to generate a standard curve to convert Bio-analyzer results from migration time to number of bases

7.3 Troubleshooting

Observation	**Suggestion**
RNA is degraded	Wear gloves throughout the procedure and when handling equipment and solutions used for RNA work. See Appendix: Preventing Sample Contamination for additional recommendations.
Final mRNA solution is too dilute for downstream applications	Purified mRNA may be concentrated by vacuum centrifugation (e.g. using a SpeedVac® concentrator).
mRNA is contaminated with excess rRNA	Ribosomal RNA may co-purify as the result of mRNA-rRNA interactions. Ensure that the total RNA is heated to 65°C prior to addition to the oligo (dT) beads and that the hybridization and wash steps are carried out at room temperature. If rRNA levels are unacceptably high for downstream applications, the mRNA preparation can be subjected to a second round of purification using fresh beads. Note that performing an additional round of purification is expected to reduce the mRNA yield.
Magnetic particles are trapped in lid or in drops on the sides of microcentrifuge tubes or stock vial	Briefly spin the tube in a microcentrifuge. Resuspend the pelleted particles by swirling or rolling the tube, or by repeated pitpetting, prior to continuing the protocol

Purified mRNA concentration measurement by optical density (OD260) or the OD260/OD280 ratio is unexpectedly low	The presence of residual magnetic particles in the purified mRNA solution interferes with measurement of optical density. Return the sample to the magnetic stand to collect the residual particles and then remeasure OD260 and OD280.
The purified mRNA solution has a brownish coloration	Brown coloration is indicative of incomplete magnetic particle removal. Return the sample to the magnetic stand to collect the residual particles and then transfer the mRNA to a fresh tube, taking care to aspirate the supernatant with a pipet tip without disturbing the magnetic particle pellet
Hybridization buffer contains a precipitate	Components of the hybridization buffer may precipitate when stored at cooler temperatures. If a precipitate is present, warm the buffer to 50°C and swirl the solution to redissolve the components

8 Vaccine Formulation with nano particles

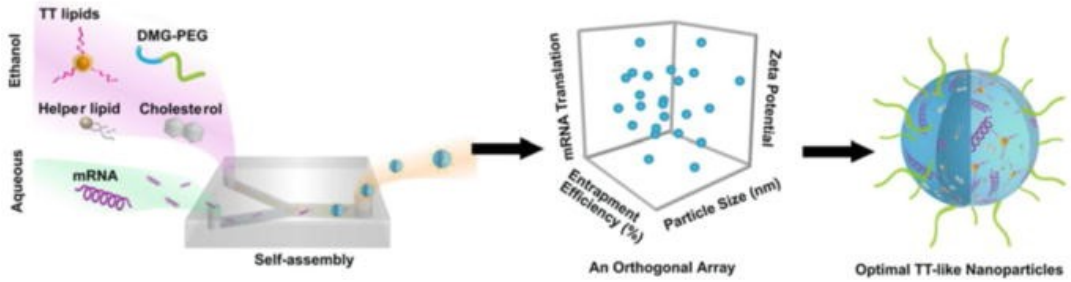
8.1 Lipid nanoparticle (LNP) formation

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5921050/

8.2 Materials

All buffers and solutions are prepared using nuclease-free water unless otherwise noted.

8.3 Preparation of Nanoparticles



1. Nuclease-free pipette tips and tubes.

2. Ethanol, 200 proof.

3. Lipid-like compounds including but not limited to TT derivates (synthesized in house according to the ref. 25). Dissolve a certain amount of lipid-like compound in ethanol to get the desired concentration (Typically, 2 mg/mL for in vitro study; 10 mg/mL for in vivo study). Keep at 4 °C.

4. Phospholipids stock solution including but not limited to 1,2-distearoylsn glycero-3-phosphocholine (DSPC), 2-dioleoylsn -glycero-3phosphoethanolamine (DOPE) and 1-palmitoyl-2-oleoylsn -glycero-3phosphoethanolamine (POPE) (Avanti Polar Lipids). Dissolve a certain amount of phospholipid in ethanol to get the desired concentration (Typically, 2 mg/mL for in vitro study; 10 mg/mL for in vivo study). Keep at 4 °C.

5. Cholesterol stock solution. Dissolve a certain amount of cholesterol in ethanol to get the desired concentration (Typically, 2 mg/mL for in vitro study; 15 mg/mL for in vivo study). Keep at 4 °C. 6. DMG-PEG2000 stock solution. Dissolve a certain amount of DMG-PEG2000 in ethanol to get the desired

concentration (Typically, 0.4 mg/mL for in vitro study; 4 mg/mL for in vivo study). Keep at 4 °C.

7. mRNAs including but not limited to firefly luciferase mRNAs (FLuc mRNA, TriLink Biotechnologies). Store at −80 °C in small aliquots.

8. Nuclease-free water.

9. 10 mM citrate buffer. Mix 41 mL of 10 mM citrate acid with 9 mL of 10 mM trisodium citrate.

10. 0.1 or 0.3 mL syringe.

11. Microfluidic mixer. LLNs used for in vivo study are prepared by fast mixing of formulation components via a microfluidic mixing device produced by Precision NanoSystems**.**

8.4 Evaluation of Nanoparticles

1. Ultrapure water with resistivity greater or equal 18.2 MΩ cm, obtainable from a water purification system such as the Milli-Q water purification system (Millipore). This is also referred to as Milli-Q water.

2. 96-well plates, tissue-culture treated.

3. 96-well opaque white plates.

4. Eagle's Minimum Essential Medium.

5. Heat-inactivated fetal bovine serum (FBS).

6. 0.05% trypsin–EDTA.

7. Quant-iT RiboGreen RNA reagent (Life Technologies).

8. Bright-Glo luciferase assay substrate (Promega; Store small aliquots at −20 °C in darkness).

9. Tris–EDTA solution (10 mM Tris–HCl, 1 mM EDTA).

10. 1% Triton X-100. Transfer 10 μL of Triton X-100 to 990 μL of Tris–EDTA buffer.

11. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) stock solution. Dissolve 0.5 g MTT in 100 mL water to obtain 5 mg/mL solution. Keep at −80 °C in small aliquots from light.

12. Dimethyl sulfoxide (DMSO).

13. Hep 3B cells.

14. Particle size and zeta potential analyzer. In this work the NanoZS Zetasizer (Malvern) has been used.

15. Microplate reader for fluorescence intensity detection (wavelength range 200– 1000 nm). In this work, the SpectraMax M5 plate reader (Molecular Devices) has been used.

16. Device for automated vitrification. In this work, a Vitrobot Mark IV system (FEI) has been used.

17. Transmission electron microscope. In this work, a Tecnai F20S/TEM (FEI) has been used.

18. Cryo-transfer holder compatible with electron microscope. For this work, a Gatan 626 cryotransfer holder (Gatan) has been used.

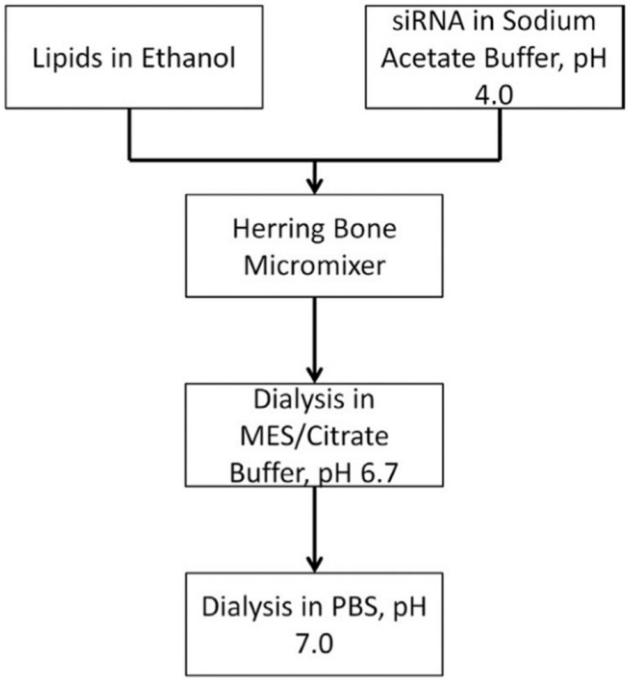
19. to sum:

mRNA encapsulation efficiency (i.e. loading efficiency) wascalculated by performing a modified Quant-iT RiboGreen RNA assay(Invitrogen) as previously described. The diameter and poly-dispersity (PDI) of the LNPs were measured using dynamic lightscattering (ZetaPALS, Brookhaven Instruments). Diameter is re-ported as the largest intensity mean peak average. LNPs preparedfor cryogenic transmission electron microscopy (Cryo-TEM) weredialyzed against 0.1x PBS, deposited onto a lacey copper grid coatedwith a continuous carbonfilm, and cooled continuously by liquidnitrogen.

8.5 Methods

8.5.1 The herringbone micromixer

The herringbone micromixer has two inlets, one for an ethanolic mixture of lipids and the other for a buffered solution of siRNA at pH 4.0. A dual syringe pump is used to drive the two streams of fluid into the herring-bone micromixer (Figure 4.6). As the two streams of fluid meet at the herring-bone micromixer, they fold and wrap around each other, exponentially decreasing the diffusion length between the two streams. This allows for rapid mixing of the two streams of fluid on a millisecond timescale. The rapid decrease in solvent polarity results in the precipitation of lipids in accordance to their



solubility.This technique has resulted in greater than 90% siRNA encapsulation efficiency over a wide range of siRNA-to-lipid ratios (Leung et al., 2012).

Fig. Encapsulation of siRNA by microfluidic mixing. This method employs a herringbone micromixer to facilitate the mixing of lipid components dissolved in ethanol and siRNA in aqueous buffer. The flow of the two streams of fluid is controlled using a dual syringe pump. The herringbone micromixer exponentially increases the surface area between the two streams of fluid, resulting in rapid mixing on a millisecond timescale. Residual ethanol is removed by dialysis and the pH of the solution is raised to 7.0. This method results in over 90% siRNA encapsulation efficiencies. Particle size is adjustable from 20 to 50 nm by varying the PEG-lipid content from 5% to 1%.

All procedures are performed at room temperature unless otherwise noted.

8.6 1 Preparation of mRNA-Loaded Lipid-Like Nanoparticles

1. To prepare x μL (the final concentration of mRNA is y μg/μL) of LLN, dissolve each component (lipid-like compound, phospholipid, cholesterol and DMGPEG2000) except mRNA separately in 200 proof ethanol in individual RNase-free tubes as stock solutions (*see* Notes 1 and 2).

2. Combine 10 xy μg of lipid-like compound with phospholipids (see Note 3), cholesterol, and DMG-PEG2000 into a 1.5 mL RNase-free tube at molar ratio 50:10:38.5:1.5 according to the ratio from ref. 30.

3. Add x /40 μL of 10 mM citrate buffer.

4. Make up to the final desired volume of x /4 μL with ethanol. If x < 1000 μL and y < 0.02 μg/μL, proceed with step 5a, b of the protocol. Otherwise, proceed with step 5c–f.

5. (a) To the above solution, add an equal volume (x /4 μL) of mRNA (xy μg) dissolved in 10 mM citrate buffer (pH = 3) by vigorously pipetting the mixture up and down (usually 50–60 times). (b) Add x /2 μL of PBS with the same procedure to form homogeneous particles (see Notes 4 and 5). (c) Transfer the above solution to a 0.1 mL syringe. (d) Afterward, dissolve xy μg of mRNA in x /4 μL of 10 mM citrate buffer (pH = 3). (e) Combine it with twofold volume (x /2 μL) of PBS and transfer to a 0.1 mL syringe. (f) Particles are then assembled through a microfluidic-based mixing device following the manufacturer's instructions (see Note 6).

6. Use freshly prepared LLNs for subsequent characterization and transfection, and store the remaining formulation at 4 °C (*see* Note 7).

8.7 Characterization of lipid-like Nanoparticles

1. Measurement of particle size and zeta potential: disperse 0.3/ y μL of LLNs into Milli-Q water to a final volume of 1 mL in disposable cuvette to make a 300 ng/mL working solution (see Note 8).

2. Determine the particle size and zeta potential of LLNs using a NanoZS Zetasizer after mixing well.

3. Measurement of mRNA entrapment efficiency: Prepare a 500 ng/mL working solution in TE buffer as described above

4. Add 50 μL of working solution (use TE buffer as blank) to each well of 96-well plate in triplicate, followed by adding 50 μL of TE buffer or 1% Triton X-100 (v/v, in TE buffer) (see Note 9).

5. To this solution, add 100 μL of 200-fold diluted Quant-iT RiboGreen RNA reagent (diluted in TE buffer).

6. Incubate for 15 min at 37 °C in the dark.

7. Measure the fluorescence on a SpectraMax M5 plate reader (Molecular Devices) with an excitation wavelength of 480 nm and emission wavelength of 520 nm. The entrapment efficiency is determined as [1 − (A TE − A blank)/(A 1% Triton − A blank)] × 100.

8. Cryo-TEM: Add approximate 3 μL of LLNs to a specimen grid.

9. Remove any excess solution.

10. Plunge the grid into liquid ethane using a vitrification device.

11. Transfer under liquid nitrogen to a cryotransfer holder that is loaded to an electron microscope.

12. Record Cryo-TEM images on a postcolumn 1k × 1k CCD camera at a magnification of 18,500× (Fig. 2).

8.8 Evaluation of Cytotoxicity of Lipid-Like Nanoparticles

1. Hep 3B cells are maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% heat-inactivated FBS.

2. Detach cells with 0.05% trypsin when they reach approximately 70% confluence.

3. Seed cells into 96-well plates (150 μL/well) at a density of 20,000 cells per well (see Note 10).

4. Treat cells with 20 μL of freshly formulated LLNs per well for 6 h after overnight culture.

5. Add 17 μL of MTT per well for 4 h.

6. Carefully remove the whole medium.

7. Add 150 μL of DMSO.

8. Dissolve MTT by shaking the plate for about 15 min.

9. Measure the absorbance using a plate reader at 570 nm.

8.9 Evaluation of LLNs-Mediated Transfection

1. Seed and treat Hep3B cells as described above except that 96-well opaque white plates are used.

2. Six hours after treatment, carefully remove the medium containing the formulation.

3. Add 100 μL of a mixture consisting of 50 μL of serum-free EMEM and 50 μL of Bright-Glo (see Note 11).

4. Cover the plate with aluminum foil.

5. Wait at least 5 min to allow complete cell lysis.

6. Measure the luminescence values using a plate reader.

8.10 Determination of the Optimal Formulation Parameters

1. Identification of lead lipid-like compound: prepare LLNs as mentioned above using newly synthesized lipid-like compounds.

2. Conduct LLNs-mediated cell transfection assay to select the lead lipid-like compound.

3. Optimization of formulation components ratio: choose a proper orthogonal array table based on the number of formulation components (factors) and assigned molar ratios (levels) (see Note 12).

4. Prepare formulation according to the orthogonal table with assigned factors and levels.

5. Conduct LLN-mediated cell transfection assays.

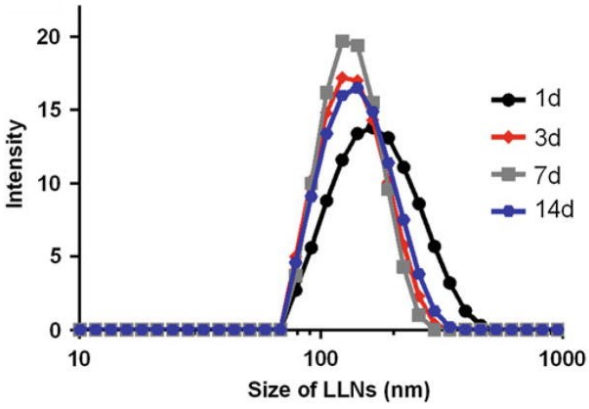
6. Predict the best ratio by analyzing the average luminescence intensity (K n) of each factor (see Note 13).

7. Readjust the intervals according to the impact trend of each component and carry out the second or even more rounds of orthogonal optimization until selecting the optimal formulation (see Note 14).

8. Optimization of the ratio for pegylation: regulate the pegylation extent of the above predicted formulation while keeping the other component ratios constant.

9. Measure LLNs-mediated luciferase expression

Examine the particle size of LLNs twice weekly (Fig. 3) to identify stable and potent formulation (*see* Note 15).



The particle stability of LLNs after incorporation of DMG-PEG$_{2000}$ (reproduced from ref. 25 with permission from (2015) American Chemical Society)

	Formulation components (molar ratio)			
Levels	Lipidoid	Phospholipid	Cholesterol	DMG-PEG$_{2000}$
1	30	1.25	18.5	0.75
2	40	2.5	28.5	1.5*
3	50*	5	38.5*	3
4	60	10*	48.5	6

Four levels assigned to each formulation component (lipidoid, phospholipid, cholesterol, and DMG-PEG$_{2000}$). *Asterisk* indicates the original molar ratio 50:10:38.5:1.5

	Formulation components (mole ratio)			
Formulation no.	Lipidoid	Phospholipid	Cholesterol	DMG-PEG$_{2000}$
1	30	2.5	38.5	3
2	40	10	18.5	1.5
3	50	10	38.5	6
4	60	2.5	18.5	0.75
5	30	5	18.5	6
6	40	1.25	38.5	0.75
7	50	1.25	18.5	3
8	60	5	38.5	1.5
9	30	1.25	48.5	1.5
10	40	5	28.5	3
11	50	5	48.5	0.75
12	60	1.25	28.5	6
13	30	10	28.5	0.75
14	40	2.5	48.5	6
15	50	2.5	28.5	1.5
16	60	10	48.5	3

Orthogonal array table L_{16} (4^4) used for optimization of formulation components ratio

8.11 Footnotes

[1]Special attention should be given when working with RNA. Assign specific benchtop and set a dedicated set of pipettes for RNA work. Spray the benchtop with RNase AWAY solution (or equivalent) to eliminate RNase contamination before preparing work solution.

[2]The concentration of each component should be adjusted according to its molecular weight. For better accuracy, ensure that stock concentration is not too high. Otherwise, the required volume is too small to be accurate; conversely, if the total volume of combined components is more than $x/4$ μL, simply increase the concentration to some extent. Typically, $x = 200$, $y = 0.01$.

[3]Store phospholipids stock at 4 °C and discard solutions exceeding one week.

[4]To minimize ethanol evaporation, close the tube cap between intervals.

[5]It is not uncommon to produce air bubbles during pipetting. Conversely, in most cases, it is an obvious feature associated with the formation of LLNs. Bubbles will disappear rapidly from solution once mixing is stopped.

[6]If a large-volume and highly concentrated LLNs are required for an in vivo study, use 3 mL syringe. It is necessary to get rid of ethanol by dialyzing samples using 1 L of 1× PBS for 60–90 min at room temperature with dialysis cassettes (3500 MWCO) before injection into animals.

[7]LLNs should be stable for over four weeks at 4 °C since no significant size change is observed during this period. Nevertheless, we recommend using freshly made LLNs.

[8]Prior to measurement, gently invert the tube several times to obtain homogenous formulations. Do not vortex or fiercely agitate the solution, which may destroy the nanostructure of formulations.

[9]Avoid creating bubbles when adding 1% Triton solution. After adding RiboGreen reagent, cover the plate with foil to avoid light since this reagent is susceptible to photo degradation.

[10]Keep equivalent cell confluence for each treatment to ensure reproducibility.

[11]Equilibrate the substrate Bright-Glo to room temperature to fully exert enzyme activity. The desired incubation time is 5–10 min to completely lysate cells and yield high luminescence signals.

[12]For the first round of optimization, large intervals between two adjacent levels are recommended in order to identify the impact trend for each formulation component. In this study, L_{16} $(4)^4$ table (Table 1) is chosen because formulation consists of four components, and each component contains four concentrations (Table 2). For simplification, interaction effects are not considered.

[13]Such a design allows rapidly predicting the top-performing formulation from 256 $(4)^4$ theoretical combinations by testing 16 combinations.

[14]Theoretically, more rounds of orthogonal optimization lead to higher potency of the predicted formulation. However, the round of orthogonal optimization depends on demand, cost, statistical difference, stability of formulation and so on.

[15]In general, pegylation hampers delivery efficiency of the formulation but significantly enhance particle stability. Consequently, the extent of pegylation should be regulated to balance delivery efficiency and particle stability, making it suitable for desired applications.

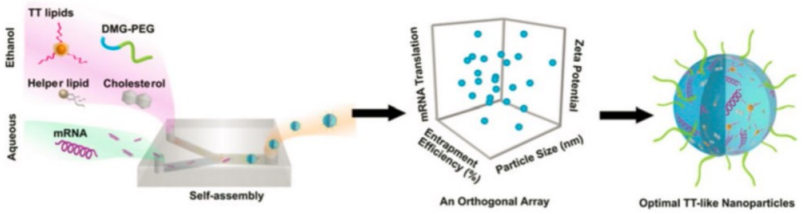


Fig. 1.
A schematic illustration of preparation and optimization of lipid-like nanoparticles for mRNA delivery (reproduced from ref. 25 with permission from (2015) American Chemical Society)

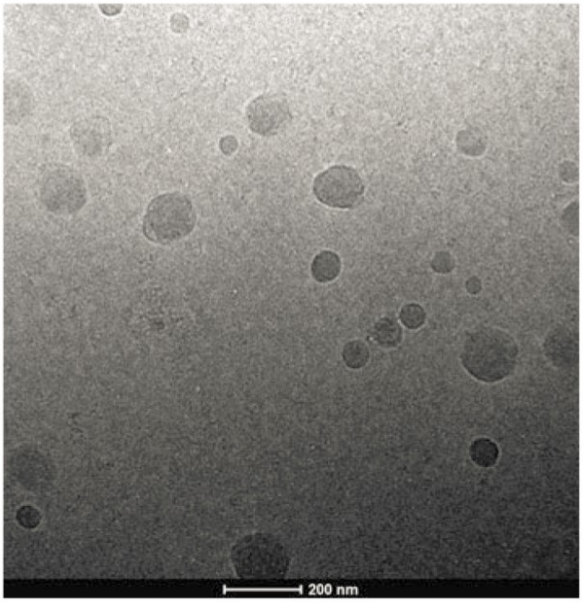


Fig. 2.
A representative Cryo-TEM image of optimized LLNs. Scale bar: 200 nm (reproduced from ref. 25 with permission from (2015) American Chemical Society)

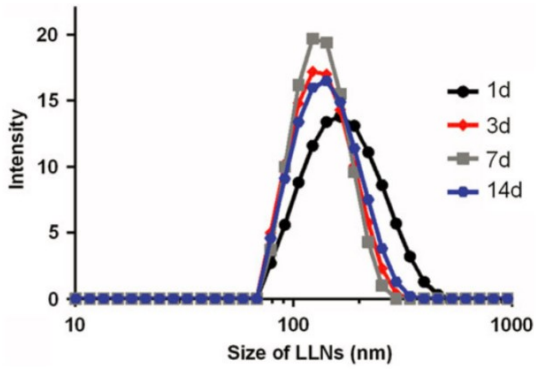


Fig. 3.
The particle stability of LLNs after incorporation of DMG-PEG_{2000} (reproduced from ref. 25 with permission from (2015) American Chemical Society)

Table 1

Four levels assigned to each formulation component (lipidoid, phospholipid, cholesterol, and DMG-PEG_{2000})

	Formulation components (molar ratio)			
Levels	**Lipidoid**	**Phospholipid**	**Cholesterol**	**DMG-PEG_{2000}**
1	30	1.25	18.5	0.75
2	40	2.5	28.5	1.5*
3	50*	5	38.5*	3
4	60	10*	48.5	6

Asterisk indicates the original molar ratio 50:10:38.5:1.5

Table 2

Orthogonal array table L_{16} (4^4) used for optimization of formulation components ratio

	Formulation components (mole ratio)			
Formulation no.	**Lipidoid**	**Phospholipid**	**Cholesterol**	**DMG-PEG$_{2000}$**
1	30	2.5	38.5	3
2	40	10	18.5	1.5
3	50	10	38.5	6
4	60	2.5	18.5	0.75
5	30	5	18.5	6
6	40	1.25	38.5	0.75
7	50	1.25	18.5	3
8	60	5	38.5	1.5
9	30	1.25	48.5	1.5
10	40	5	28.5	3
11	50	5	48.5	0.75
12	60	1.25	28.5	6
13	30	10	28.5	0.75
14	40	2.5	48.5	6
15	50	2.5	28.5	1.5
16	60	10	48.5	3

8.12 NanoZS Zetasizer

8.12.1 Important Notification:

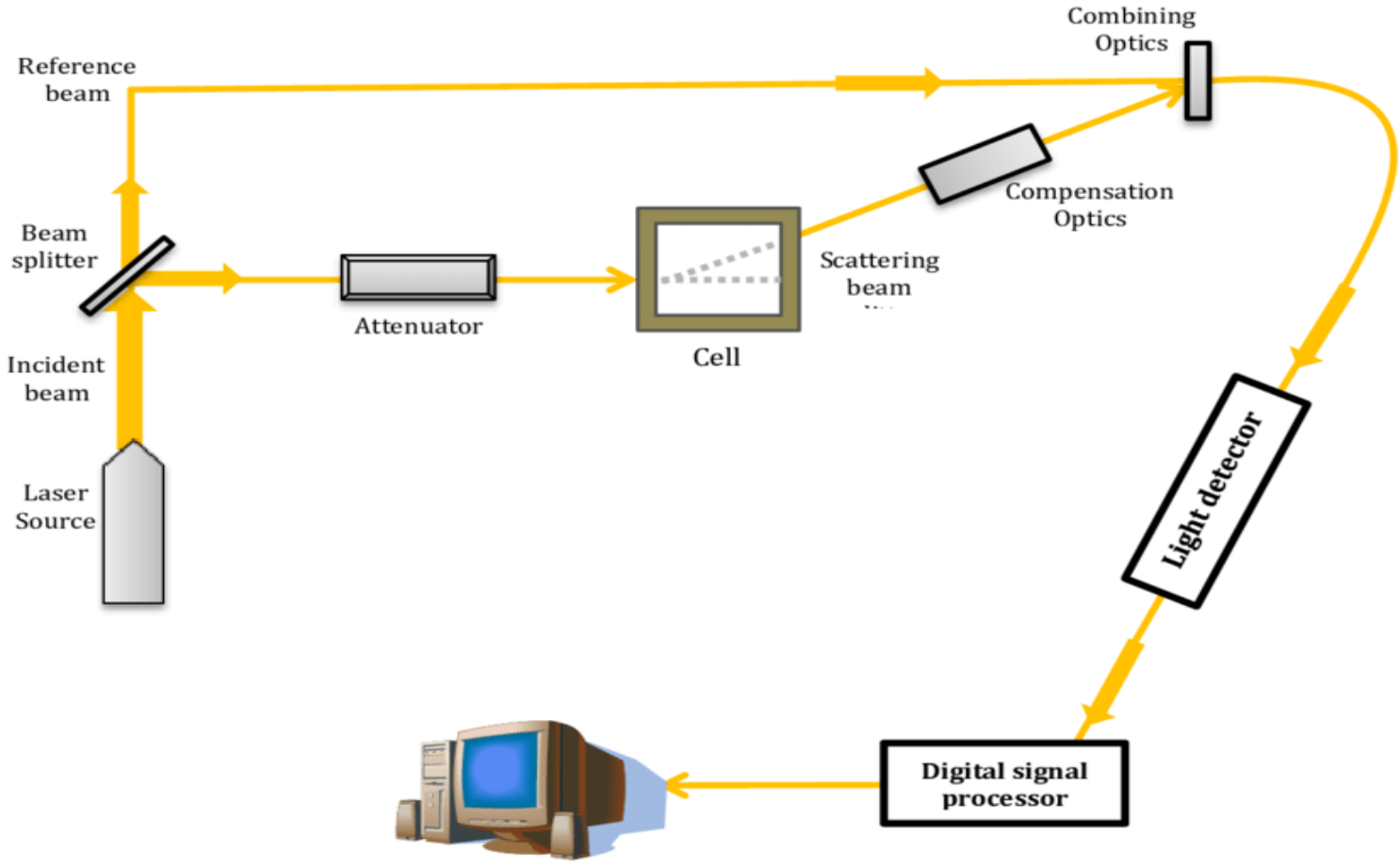
* (2010-04-02) Cuvettes should be rinsed and sonicated in your buffers and/or ddH2O before applying samples for measurements. (2009-09-02) Please avoid dusts or contaminate when preparing your sample. Do not transfer samples from container to container, which introduces dusts and bubbles.

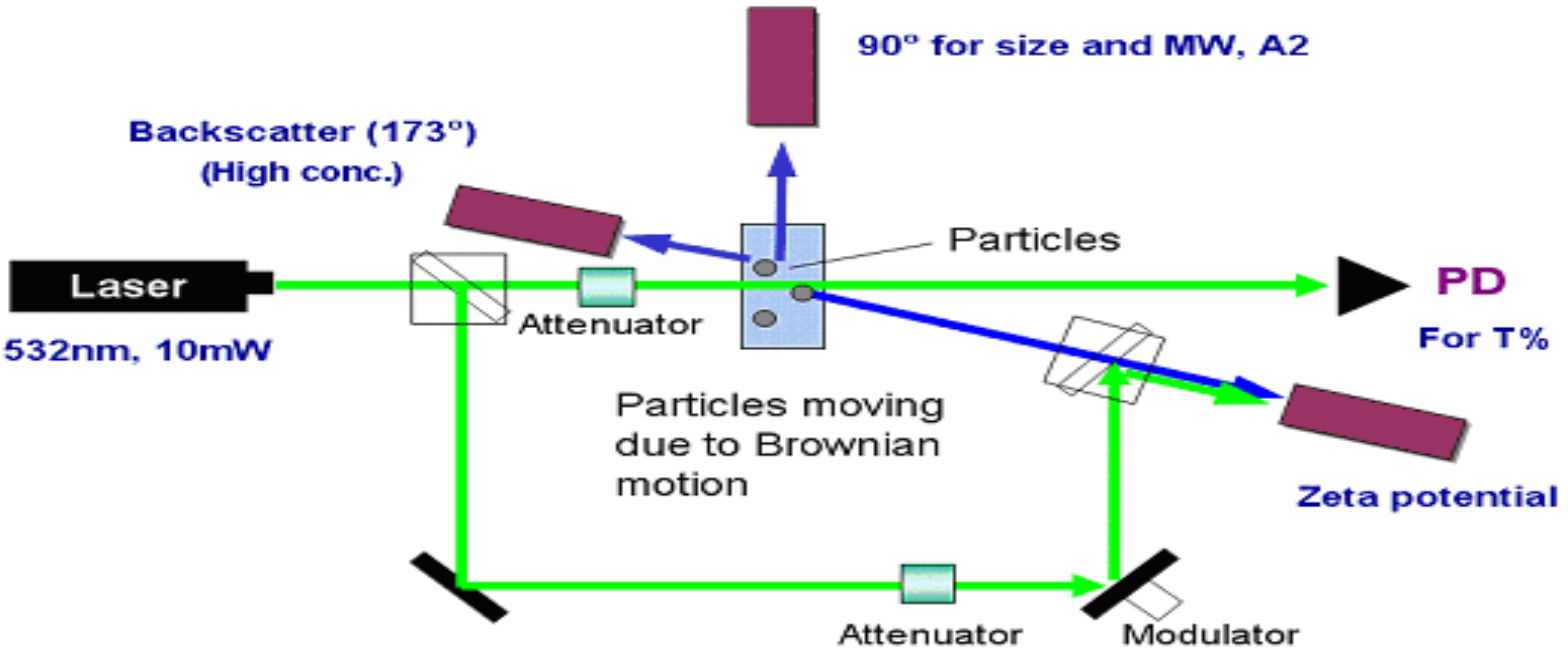
8.12.2 Specification

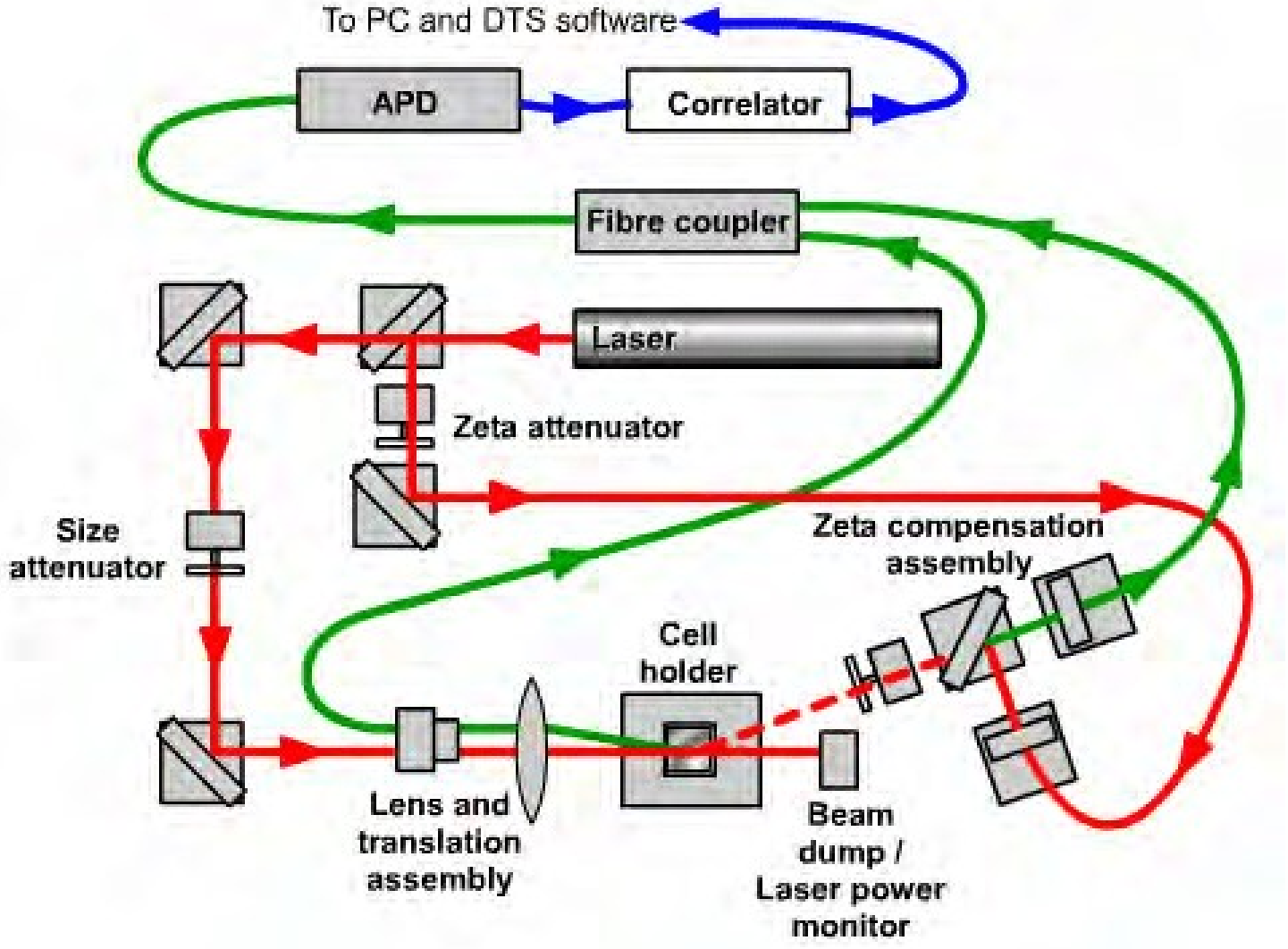
Laser wavelength	633nm
Particle size range	0.6nm - 6μm
size range for zeta potential	5nm - 10μm
size range for Mw	1000 - 2x10^7 Da
sample volume	100 μl
Temperature range	2-90 ℃

8.12.3 Recommended Sample concentration

Particle size	Min. concentration	Max. concentration
< 10 nm	0.5g/l	----------
10nm - 100nm	0.1g/l	5% mass
100nm - 1μm	0.01g/l	1% mass
> 1μm	0.1g/l (0.01%	1% mass


Combining Optics
Reference beam
Compensation Optics
Beam splitter
Scattering beam
Attenuator
Cell
Incident beam
Light detector
Laser Source
Digital signal processor


90° for size and MW, A2
Backscatter (173°)
(High conc.)
Particles
Laser
532nm, 10mW
Attenuator
PD
For T%
Particles moving
due to Brownian
motion
Zeta potential
Attenuator
Modulator


To PC and DTS software
APD
Correlator
Fibre coupler
Laser
Zeta attenuator
Size attenuator
Zeta compensation assembly
Cell holder
Lens and translation assembly
Beam dump / Laser power monitor

9 Appendix: Vitamin C encapsulated NLC protocol

This protocol produces one batch (5 mL) of NLC dispersion. It can be replicated in all suitably equipped laboratories.

Pre-work: For each sample batch of NLC, require 2 clean test tubes (2 cm diameter) and one beaker (50 mL size) with magnetic stirrer (2.5 cm in length). Glassware must be cleaned with ethanol and soapy water and dried before use.

9.1 Method

Weighing and processing of raw ingredients into Eppendorf/ glassware as follows:

Drug Extract in Eppendorf	Lipid test tube	Surfactant test tube 2	1 Final Beaker + magnetic stirrer
250 mg Vitamin C (or nothing if no drug)	125 ± 3 mg oleic acid (approx. 8 drops with P1000 set to 200 µL)	30 ± 0.5 mg PVA (use a metal spatula to carefully transfer)	
850 µL type II water (use a P1000 pipette)	500 ± 4 mg Witepsol® E85 (weigh on foil and transfer)	270 ± 5 mg Tween 80 (about 10 drops with P1000 set to 1000 µL; adjust with a fresh pipette tip if required).	
	1 mL dichloromethane (DCM) solvent (fully depress micropipette plunger) – cover with parafilm immediately	2925 µL type II water (if no drug, = 3025 µL water) – cover with parafilm	
Vortex mix. 2-3 mins to ensure full dissolution	Sonicate 2 mins (agitate in ultrasonic cleaning bath by moving test tube around)	Vortex mix 2 mins Sonicate 2 mins in ultrasonic cleaning bath Vortex mix2 mins To ensure full dissolution	Place magnetic stirrer in beaker.

Set up of equipment:

• A **sonicator** (Vibra-CellTM ultrasonic processor, Sonics & Materials Inc., Newtown USA) equipped with a 6 mm sonication probe is required. The sonicator was set to 70% amplitude.

• A multiposition magnetic stirrer plate (IKA® RO 15 magnetic stirrer, IKA-Werke GmbH & Co (Staufen, Germany)). The stirrer plate was set to a speed of 300 rpm.

• Stopwatch (to measure ultrasonication time)

9.2 NLC preparation step-by-step procedure:

1	Clean ultrasonication probe with tissue and Ethanol (and in between samples). Make sure beaker + magnetic stirrer is pre-weighed.
2	Take a 100 mL beaker, fill with ice and water (to the 60 mL mark) and place directly underneath the ultrasonication probe.
3	Using a P1000 pipette, transfer all of the drug extract (P1000 pipette set to 1000 µL) into the lipid test tube 1
4	Move the probe into the middle of the sample in lipid test tube 1. It may be helpful to use the indication line (in black marker – indicated on the clamp) to assist with set up Ultrasonicate for 3 mins at 70% amplitude. Use a stopwatch. (Primary emulsion will appear white, listen for the sound and observe thorough mixing)
5	Remove lipid test tube 1 and transfer contents to surfactant test tube 2 as quickly as possible and as much as possible.
6	Now place the surfactant test tube 2 under the ultrasonication probe. Ultrasonicate for 3 mins at 70% amplitude. Use a stopwatch. Ensure thorough mixing of the dispersion and listen to the sound. The Secondary emulsion is homogenous and bright white in appearance.
7	Transfer the secondary emulsion fully to the beaker. Record the weight of the beaker immediately.
8	Note the start and end time of DCM evaporation. Place the beaker on magnetic stirrer bar at 300 rpm. Cover the side of the beaker with foil

	(height of foil = parafilm height). The fume cupboard light should be turned off. This will take approximately 2 hrs 30 min. This time should be kept the same for all replicates.
9	Calculate the water that was lost by evaporation and add the required quantity to the NLC dispersion. Put NLC dispersion back on the stirrer bar, covered, for 2 minutes to ensure full mixing. (Use a P200 pipette and type II water)
10	Weigh an empty and labelled storage vial. Transfer formulation to the vial. Use a magnet to remove the magnetic stirrer. Reweigh the vial.
11	Calculate 1% by weight of preservative. Add preservative (Use a P10 pipette, only dispense amounts of 5 to 10 uL carefully on the weighing balance). DO NOT GO OVER 1%. Vortex mix the formulation. Record the actual weight added. (if making NLCs with free drug (not encapsulated), then add 5% vitamin C here and vortex mix for 2 minutes thoroughly)
12	Adjust the pH of the formulation. Add approximately 4.39967 mg of NaOH. 8.0M NaOH can be made by dissolving 320 mg NaOH / mL of type II water in a volumetric flask. Add approximately 13.75 µL of 10.0M NaOH to raise the pH to above 3. Add dropwise with mixing in-between. Vortex mix for 1 min. Take an aliquot in an Eppendorf to measure pH.

Preparation additional notes:

- Use the ice bath only for 2 samples (max 12 minutes sonication time)
- Align the ultrasonication probe vertically with the indication line on the clamp and ensure complete ultrasonication.
- If making free drug NLC, add vitamin C at the very end (when in storage bottle). Weight on foil and scrape foil with metal spatula (5% by weight by difference)

Weight recording table:

	NAME	Weight (approximately)	NOTES
A	Empty beaker + stirrer	34.99g	

B	Beaker + stirrer + NLC (before evap)	40.75g	Amount of DCM = 1 mL X 1.33 g/mL = 1.33 g
C	Beaker + stirrer + NLC (after evap)	39.1663g	
D	Total evap losses [= B – C]	1.5846g	
E	Total water losses [= D – 1.33]	0.2546 g (= 254.6 uL)	
F	Empty vial weight	14.8349g	
G	Empty vial + NLC dispersion weight	19.0681g	
H	Weight of NLC dispersion [= G – F]	4.2332g	
I	Max weight of preservative (1%) (if Free Drug NLC, add 5% AA here)	42.332g	This is 1% of H
J	Actual weight of preservative added	e.g. 37.6 mg	

pH before NaOH addition: _____

Volume of 1.0M NaOH addition: _____

pH after NaOH addition: _____

Storage: wrap in foil and store in the fridge at 4 oC

9.3 Measurement of particle size and zeta potential of SLN and NLC

The size and zeta potential of SLN were measured by photon correlation spectroscopy using a Zetasizer 3000 HSA (Malvern, UK). Samples were diluted appropriately with the aqueous phase of the formulation to get optimum kilo counts per second (Kcps) of 50-200 for measurements, and the pH of diluted samples ranged from 6.9 to 7.2. Zeta potential measurements were carried out at 25°C, and the electric field strength was around 23.2 V/cm.

10 SLN Protocol 2

https://www.americanpharmaceuticalreview.com/Featured-Articles/565893-Solid-Lipid-Nanocarrier-Development-Toolbox-for-Increasing-Oral-Bioavailability-of-API/

a brief review on solid lipid nanoparticles: part and parcel of contemporary drug delivery systems

https://pubs.rsc.org/en/content/articlehtml/2020/ra/d0ra03491f

11 SLN Preparation Techniques

Table 1. Advantages and drawbacks of commonly used methods to produce SLN and NLC

Preparation Technique	Advantages	Disadvantages
Emulsion diffusion	• Room temperature • Larger choice of solvents than nanoprecipitation • Small particle size	• Solvent-based process • Very diluted nanosuspensions • High energy process
Emulsion evaporation	• Low energy process • Small particle size and PDI	• Solvent-based process • Time consuming process
Gasco method	• Solvent-free process • Low energy process • No specific equipment required	• Diluted nanosuspensions
Cold HPH	• Solvent-free process • Low cost process • Reduced exposition of molecules to high temperatures	• Risk of API partition in the aqueous phase (for BCS class III drugs) • Broad particle size distribution
Hot HPH	• Easily scalable process • Solvent- free process • Small particle size and low PDI • Low cost process	• Risk of drug degradation induced by high temperature, heat transfer to the formulation or high shear rates
Nanoprecipitation	• Room temperature • Low energy process • Rapidity of particle production	• Solvent-based process
SFEE	• Low temperature • Solvent with low toxicity (carbon dioxide)	• Risk of API degradation under high energy process
Ultrasonication	• Solvent-free process • One step process • Small particle size	• High temperature • Heat transfer to formulation • Laboratory scale • Risk of metallic contamination of the samples

11.1 Preparation of Solid-Lipid Nanoparticles.

The SLN were prepared from a warm oil-in-water (o/w) microemulsion byusing Compritol ATO 888 and DDAB as the lipid matrix. Briefly, 0.273 mmol of Compritol were heated to 10°C aboveits melting point and mixed with 2.5 mL of a hot aqueoussolution of Pluronic F68 (0.158 mmol) and DDAB (1.45 mmol)to form a clear microemulsion, under mechanical stirring. Then,cationic nanoparticles were obtained by dispersing undermechanical stirring at 1000 rpm the warm o/w microemulsionin cold water (2-3°C) (organic/aqueous volume ratio equal to1:10). The obtained cationic nanoparticles were purified by dialysis using a Visking Tubing Dialysis 18/32″(with amolecular weight cutoff of 12 000-14 000 Da). Then, cationicnanoparticles were freeze-dried using a Modulo freeze-dryer(Labconco Corporation, Missouri). To label SLN, fluoresceinfree acid was added to the lipid phase during preparation of themicroemulsion.

SLN or NLC may be obtained by a variety of formulation techniques adapted to the properties of the API such as solubility, pKa, heatsensitivity, shear-sensitivity, and dose requirements.

A coarse, hot emulsion generally formed by high shear homogenization is poured into the reservoir of the HPH system. The coarse emulsion is forced through an interaction chamber consisting of a narrow gap (few microns) of predefined shape (represented in Figure 1), under high pressure (100 to 2000 bars). The high shear rates resulting from the impact (collision) forces of the walls and the particles themselves enable significant size reduction.[7,8] Several passages in the interaction chamber are generally required to obtain a monodisperse hot nanoemulsion. A recent article describes the different formulation step to formulate NLC for lipidized peptides encapsulation.[9]

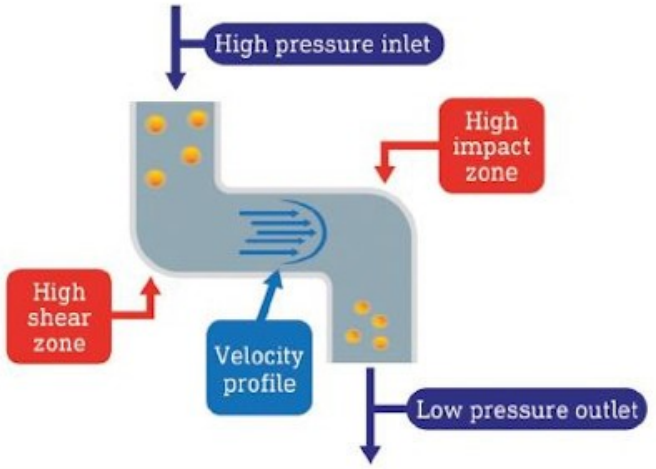


Figure 1. Schematic representation of the phenomenon happening in the interaction chamber of HPH

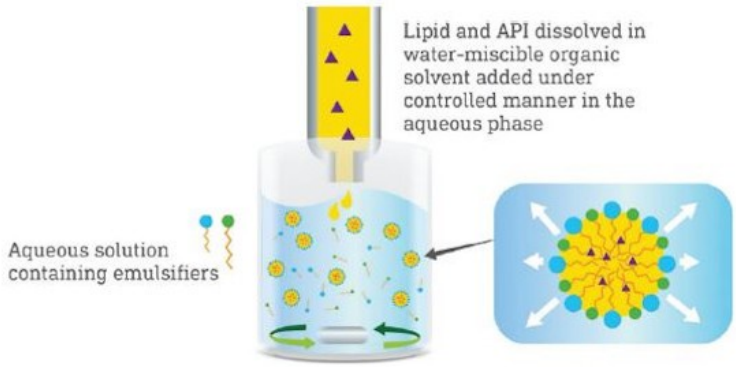


Figure 2. Schematic representation of nanoprecipitation method to formulate SLN and NLC

Whether by ultrasonication or hot HPH, most of the energy provided to the system is dissipated by Joule effect. Consequently, the formulation temperature rapidly increases which can potentially result in API degradation. For thermosensitive API, cold HPH process was developed. With this technique,

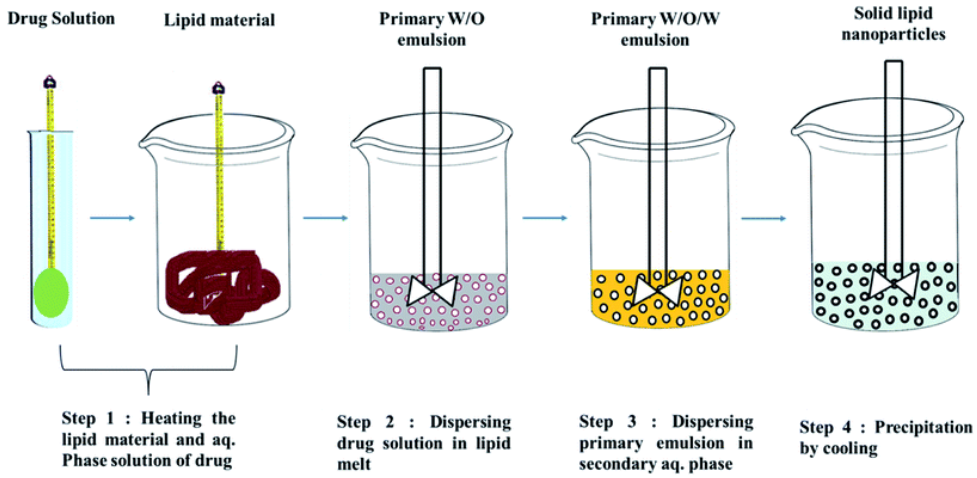
the API is also dispersed or dissolved in the molten lipid phase which is then rapidly cooled by use of liquid nitrogen or dry ice. This material is then milled into microparticles and suspended in a cold emulsifier solution before injection into the HPH.

Although the processes described above are easy to set up, they may be deleterious to shear sensitive drugs such as DNA, albumin or erythropoietin.[10] For these API, low energy melt dispersion processes such as membrane contactors can be used. With the latter, the molten lipid containing the API is continuously pushed through the membrane contactor of defined pore-size. The nanodroplets are formed at the surface of the membrane where an aqueous solution of emulsifier(s) circulates and sweeps away the particles.[11] Particle size will depend on the type of lipids, the process temperature, membrane pore size, and the flow velocity of the aqueous phase.

11.2 Preparation method

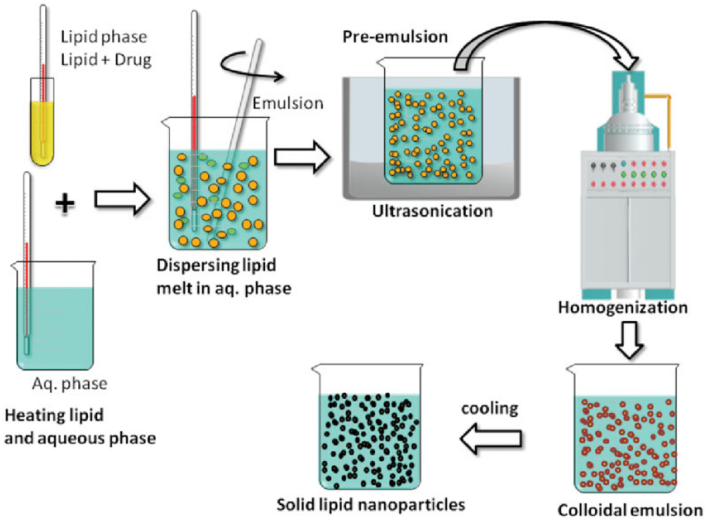
11.2.1 Water/oil/water (w/o/w) double emulsion method.

Water/oil/water (w/o/w) double emulsion method. This method is mainly used for the preparation SLNs loaded with hydrophilic drugs and some biological molecules such as peptides and insulin. SLNs are produced from w/o/w multiple emulsions via the solvent in water emulsion diffusion technique, insulin is dissolved in the inner acidic phase of the w/o/w multiple emulsion and lipids dissolved in the water-miscible organic phase, and then SLNs are produced by diluting the w/o/w emulsion in water. This results in the diffusion of the organic solvent into the aqueous phase and precipitation of the SLNs. The nature of the solvent and interaction of the hydrophilic drug with the solvent and excipients affect the preparation process using this method.



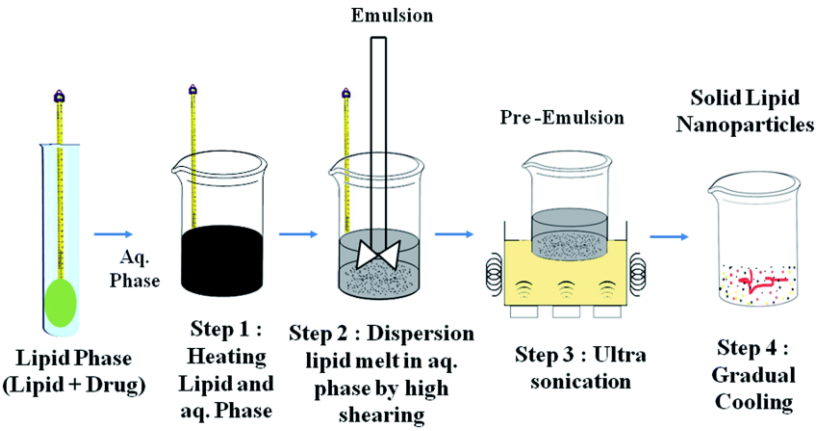
11.2.2 High pressure homogenization or HPH (hot/cold)

. HPH is a technique in which high pressure (100 to 2000 bar) is used to push a liquid or dispersion through a gap of few micrometers to produce submicron size particles. A high shear stress and cavitational forces break down the particles, resulting in a decrease in particle size. HPH can be performed either at high temperature or below room temperature, called hot-HPH and cold-HPH, respectively (Fig. 3).[70] In the first step of both processes, the lipid(s) and drug(s) are heated to about 5–10 °C higher than the melting point of the lipid so that the drug is dissolved or dispersed in the melted lipid.[71] Generally, the concentration range of lipid is between 5% to 20% w/v. In the second step of the HPH technique, the aqueous phase containing the amphiphile molecules is added to the lipid phase (at the same temperature as the lipid melting) and the hot pre-emulsion is obtained using a high-speed stirring device. The lipid (more added for homogenization) is forced at high pressure (100–1000 bar) through a narrow space (few μm) for 3–5 times, which depends on the formulation and required product. Before homogenization the drug is dispersed or dissolved in the lipid melt. However, there are certain drawbacks to this method as follows: (1) it cannot be used for heat-sensitive drugs because of their degradation and (2) an increase in the number of rotations or pressure of homogeneity often results in an increase in particle size.[72] However, these limitation can be overcome using cold-HPH to prepare SLNs. As discussed earlier, the first step involves the formation of a suspension of melting lipids and drugs, followed by rapid cooling in dry ice and liquid nitrogen. In the third step, the powder is converted into micro-particles by milling. Then, the micro-particles are dissolved cold aqueous surfactant solution. In the last step, to create SLNs, homogenization is usually performed for 5 cycles at 500 bars.[73]



11.2.3 Ultrasonication

This method is based on the principle of particle size reduction by applying sound waves. In this method, homogenization with high pressure and ultrasonication are simultaneously used to prepare SLNs with a size in the range of 80–800 nm



11.3 Nanocarrier Composition

A typical LNP contains four parts, the ionizable lipid that allows the LNP to self-assemble, the stabilizing agent (cholesterol), a phospholipid that lends stability to the bilayered encapsulating lipid structure, and a stabilizing lipid-containing polyethylene glycol (PEG) that promotes stability.

Triglycerides, waxes, partial glycerides, and fatty acids of varying chain length or ester content constitute the "lipids" used in SLN and NLC. Generally, the higher the percentage of the formulation lipids, the larger is the size of the particles obtained. Moreover, an increase in the chain length of the lipids increases the viscosity of the lipid phase in melt processes. Consequently, higher shear rates are required with long lipid chains to reduce particle size. High levels of emulsifiers are needed to stabilize the SLN formation process where high shear rates create numerous interfaces among the particles. All types of emulsifiers may be used for this purpose which have been listed in a 2012 review by Mehnert et al.

In the case of NLC, a liquid lipid fraction is added to the system to increase the mobility of the chains upon lipid crystallization. It can also be used to increase intestinal permeability, especially in the case of medium-chain triglycerides,[18] or for their protective effect towards proteolytic degradation in the case of peptides.[19] The liquid lipid should be sufficiently hydrophobic to remain in the nanoparticles, otherwise an exudation phenomenon may be observed.

11.4 Characterization of Lipid Nanoparticles *ParticleSize*

Physical and chemical characterization are also required after the preparation of SLNs. Due to the particle size, complexity and dynamic nature of the delivery system, the characterization of SLNs is a serious challenge. The parameters needed to evaluate SLNs include particle size, zeta potential, degree of crystallinity, drug release, entrapment efficiency (% EE) and surface morphology. Particle size, polydispersity index and charge analysis can be measured by photon correlation spectroscopy (PCS), dynamic light scattering (DLS) and quasi-elastic light scattering (QELS).[93] The main advantage of these techniques is that they are not time-consuming, with speedy analysis and high sensitivity.[94] The crystallinity of lipid or polymorphic modifications can be

analyzed via differential scanning calorimetric analysis (DSC).[95] The crystallinity within nanoparticles is measured by the function of the glass and melting point temperature associated with the enthalpies. Nuclear magnetic resonance (NMR) can also be used to determine the size and qualitative nature of nanoparticles. Changes in their chemical shift are related to the molecular dynamics, which provide information about the physicochemical state of the constituents inside the nanoparticles. Electron microscopy is an advanced technique that can offer a direct way of observing nanoparticles. The size, surface topography, stability and structural changes of SLNs with time can be better investigated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). However, cryo-microscopic analysis involves rapid freezing, and thus the specimen is preserved in its hydrated state. Cryoelectron microscopy (cryo-EM) such as cryo-TEM and cryo-FESEM provides 3D images of stable frozen-hydrated particles.[96] Atomic force microscopy (AFM) is more advanced than TEM and SEM. This method allows atomic-level resolution to be accessed together with size, colloidal attraction and resistance to deformation, making AFM an important tool. The surface distribution of surfactant molecules, bio-conjugation confirmation in case of cationic SLNs, and functionalization of nanoparticles can be estimated by X-ray photoelectron spectroscopy (XPS).[97,98] SLN entrapment can be measured by either centrifugation or micro-centrifugation techniques. The samples are centrifuged at high rpm, and the amount of free compound is determined by UV-Visible spectroscopy or high-performance liquid chromatography in a clear supernatant.[99–101] The drug loading and release profile or release kinetics of SLNs depend on the crystalline state and melting behavior of the lipid.[10]

The functionality of nanoparticles is largely correlated with particle size mainly because the latter impacts drug release and absorption rate at the intestinal barrier that is one of the main obstacles to oral bioavailability. Particle size measurements also involve determination of the polydispersity index (PDI) which expresses the relative error between curve fit and experimental values, giving an overview of the quality of the dispersion and guaranteeing a homogeneous repartition of the API within the formulation and a good stability of the system.

Laser Diffraction (LD) and Dynamic Light Scattering (DLS) are commonly used techniques for measuring particle size. LD is based on Fraunhofer's theory that relates the diffracted light intensity and the diff raction angle to the particle size (e.g. the smaller the particle the larger the angle of diffraction). A clear advantage of the LD technique is that it covers a wide range of sizes, from a few nanometers up to one millimeter. DLS, also called Photon Correlation Spectroscopy (PCS) on the other hand is based on Rayleigh diffusion, which states that light diff uses in all directions as long as the particles are small compared to the wavelength (generally 633 nm). The laser goes through all particles in Brownian motion which causes fluctuation in scattering intensities. Constructive and destructive interferences are generated, giving information about particle speed and consequently particle size (larger particles display slower movements). A less common technique to measure particle size in a more accurate manner is the combination of Field Flow Fractionation (FFF) with MultiAngle Light Scattering (MALS) which enables the separation and particle size analysis of several populations in polydispersed nanosuspensions.20

All the techniques mentioned above provide a single value to characterize particle size. However, only perfect spheres can be described by a single number. Consequently, the values obtained for non-spherical objects correspond to the diameter of an equivalent sphere that has one property in common with the analyzed particles (equivalent volume, equivalent weight,

minimum diameter, etc. Although all responses will be correct, the values will vary as a function of the selected property. It is then important to compare several techniques to obtain a more complete description of the system. The ideal solution is to complete the particle size analyses by observations of the morphology of the particles.

11.5 Morphology Assessment

Assessment of particle morphology is especially important in the case of solid lipid nanocarriers for which platelet structures are commonly observed upon lipid crystallization.[10] The following are widely used microscopic tools for evaluating nanoparticle morphology.

Transmission Electron Microscopy (TEM) facilitates 2-D observations on interactions between the electrons transmitted to a thin sample and the material, providing a very high-resolution image. A prerequisite for this analytical technique is that the sample be electron transparent. Although this method is considered to be non-destructive, the electron beam can cause melting of the lipid nanoparticles hence affecting their structure and integrity. To preserve their morphology, it is therefore recommended that the analyses involve cryo-TEM whereby samples are frozen in liquid ethane prior to observations. Examples of SLN and NLC observations by cryo-TEM are provided in Figure 3. Scanning Electron Microscopy (SEM) provides information about the surface morphology and 3D structures of nanoparticles. Whereas TEM utilizes electron transmission through the sample, SEM measures electron transmission of the sample surface. A beam of high-energy electrons is emitted to generate different signals at the surface of the sample. Secondary electrons provide high resolution SEM images. Backscattered electrons (BSE) and diffracted backscattered electrons (EBSD) are used to determine crystal structures and orientations, as well as the photons, visible light and heat. An additional step for sample preparation may involve electrically insulating the samples by coating them with a thin layer of conducting material. For the same reasons as for TEM, it is recommended to cryogenically fix the samples before observation to maintain their structure under the electron beam.

Other less common methods can be used to evaluate particles morphology. In Cryo-Field Emission Scanning Electron Microscopy (cryo-FESEM), samples are frozen in liquid nitrogen and the measurements are conducted in the frozen state. Atomic Force Microscopy (AFM) technique provides high resolution (0.01 nm) 3-D topography. With this technique, samples can be directly

analyzed; conductivity of the material is not required and the measurements can be realized in presence of solvents (no vacuum necessary)

11.6 Zeta-potential

Zeta-potential, or ζ-potential, represents the electric charge of the nanoparticles. It is measured by electrophoresis, a technique that identifies the movement of the charged nanoparticles in suspension under a magnetic field. Zeta-potential is a useful measure for predicting the stability of SLN in suspension as it relates to the tendency of the nanoparticles to repulse each other as particle electric charge increases. In oral delivery, Zeta-potential is also a tool to predict the intestinal absorption of the nanoparticles as neutral and anionic ones are more likely to cross the mucus barrier than positively charged nanoparticles.

Other characterization tools such Differential Scanning Calorimetry (DSC), X-ray diffraction or Proton-NMR can be used to study the inner structure of the particles and predict their stability and behavior.

11.7 Stability issue and storage conditions of SLNs

It has already been reported that SLNs are stable for more than three years. The stability of SLNs is mainly associated with their lipid material, surfactant concentration, and temperature optimization during their preparation. Thus, all these parameters should be considered for their stability and storage. Triglycerides undergo α (alpha), β (beta) and β′ (beta prime) crystal modification during their preparation and storage. The kinetics of their polymorphic transitions largely depend on their chain length, where the crystallization process is slower for longer chain than shorter chain triglycerides. Sometimes SLNs undergo gel formation, and their gelling tendency strongly depends upon β′ modification due to exposure to light, temperature and shear force.[7] Also, the size of the particles can vary because of exposure to light.

In a study, SLNs were exposed to various destabilizing factors, and it was found that gelation occurred and their zeta potential decreased. However, SLNs have several stability issues and the drug may be hydrolyzed in aqueous dispersion. Thus, drying is a necessary option for the prolonged storage of SLNs. Freeze drying, spray drying, and lyophilization are techniques for drying. Recently, the electrospray method was employed to prepare SLNs, where a dry SLN powder was obtained directly. The formulation of SLNs in a powder form, which may be loaded into pellets, capsules, or tablets, makes these materials highly advantageous for drug delivery. On the other hand, the applications of SLN formulations may be restricted due to their uncontrolled particle growth

through coagulation or agglomeration, generating very swift "burst release" of the drug. SLNs possess perfect crystal lipid matrices, which carry the loaded drug in its molecular form between fatty acid chain-formation and uncontrolled, unwanted enhancement of the crystal structure during both the production and storage of SLNs often result in the release of the loaded drug solution, which is a huge drawback of SLNs.

11.8 Encapsulation Efficiency

The amount of API actually encapsulated in the nanoparticles is the most important measure of the process efficacy. It is calculated by subtracting the quantity of free drug in the supernatant from the overall amount of API in the nanosuspensions as follows:

Equation 1: Encapsulation efficiency calculation:

$$Encapsulation\ efficiency, \% = \frac{Total\ amount\ of\ API\ in\ the\ formulation - Amount\ of\ free\ API}{Total\ amount\ of\ API\ in\ the\ formulation}$$

Drug loading is calculated after drying the nanoparticles and corresponds to the ratio of API within the nanoparticles:

Equation 2: Drug loading calculation

$$Drug\ loading, \% = \frac{amount\ of\ drug\ encapsulated}{weight\ of\ nanoparticles}$$

The total amount of API can be easily quantified after dissolution of the nanoparticles in an appropriate solvent. Measuring the amount of API in the supernatant is trickier as it requires a proper separation from the nanoparticles. A number of methods like ultrafiltration, field flow fractionation, and ultracentrifugation have been described in the literature. Lv et al. recently compared three commonly used techniques to separate nanoparticles from their dispersing environment. The study reported poor separation with centrifugation, especially with SLN, despite the high centrifugal forces applied. Gel permeation chromatography showed good separation of large

macromolecules although the latter may adhere to the beads constituting the column, resulting in distortion of the results. Finally, the filtration/centrifugation method using membrane with pre-defined mesh size offered good separation of the nanoparticles, with only 0.32-0.93% drug leakage from the nanoparticles.

11.9 Intestinal Permeability

The intestinal barrier consists of a mucus layer and the intestinal epithelium. Mucus is a complex hydrogel, negatively charged and prevents the passage of large molecules. The epithelium is mainly composed of enterocytes (99%) with hydrophobic phospholipid bilayers that prevent the passage of hydrophilic molecules. These cells are covered with microvilli and are separated by tight junctions which limit the transcellular passage to small molecules. The other cells constituting the epithelium are endocrine cells (or L cells), Goblet cells responsible for the production of mucus and M cells known to initiate mucosal immunity responses. These latter cells are only present in specific regions known as Peyer's patches. They are less covered in mucus and microvilli and are more conducive for the passage of large molecules. Once absorbed, the macromolecules are conducted to the lymphoid system. This absorption route presents the advantage of avoiding the first pass effect. Moreover, M cells are not restricted to small molecules so as enterocytes (limited to 50-100 nm).

11.10 In-vitro cell models

Caco-2 cells, Madin-Darby Canine kidney (MDCK) cells and Parallel Artificial Membrane Permeability Model (PAMPA) are the common in vitro models used in the evaluation of intestinal permeability of API and pharmaceutical preparations. In the case of PAMPA, an artificial lipid membrane is formed by a mixture of lecithin and an organic solvent. This technique is cost-effective and easy to set up which can be of great advantage in the early formulation development phase. However, its use is limited to the evaluation of passive transport.

MDCK cells use is less commonly reported than that of Caco-2 cells, although they are said to enable a more predictive intestinal apparent permeability and more inter-laboratory reproducibility than Caco-2 cells.

12 DLS particle size measurement

Small particles in suspension undergo random thermal motion known as Brownian motion. This random motion is modeled by the Stokes-Einstein equation. Below the equation is given in the form most often used for particle size analysis.

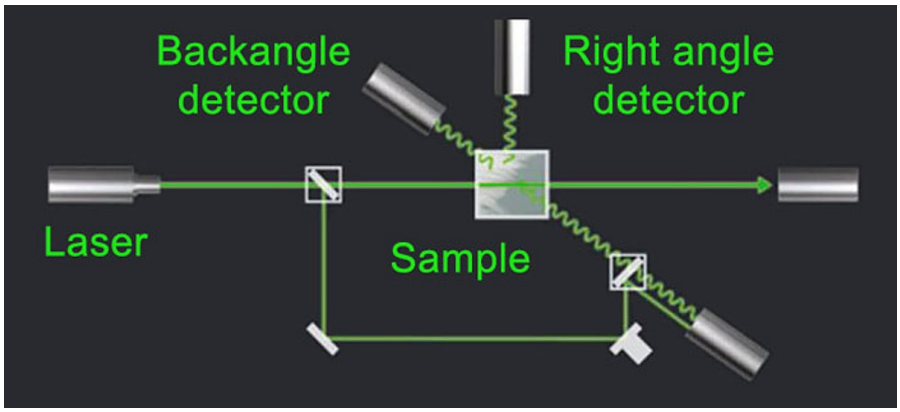
$$D_h = \frac{k_B T}{3\pi\eta D_t}$$

where

- D_h is the hydrodynamic diameter (this is the goal: particle size!)
- D_t is the translational diffusion coefficient (we find this by dynamic light scattering)
- k_B is Boltzmann's constant (we know this)
- T is thermodynamic temperature (we control this)
- η is dynamic viscosity (we know this)

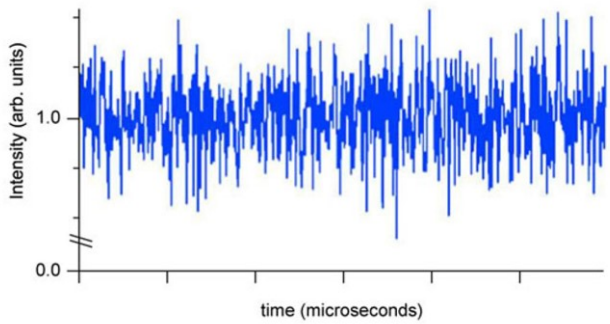
The calculations are handled by instrument software. However, the equation does serve as important reminder about a few points. The first is that sample temperature is important, at it appears directly in the equation. Temperature is even more important due to the viscosity term since viscosity is a stiff function of temperature. Finally, and most importantly, it reminds the analyst that the particle size determined by dynamic light scattering is the hydrodynamic size. That is, the determined particle size is the size of a sphere that diffuses the way as your particle.

12.1 Measuring Particle Motion I: Dynamic Light Scattering Optical Setup



Light from the laser light source illuminates the sample in the cell. The scattered light signal is collected with one of two detectors, either at a 90 degree (right angle) or 173 degree (back angle) scattering angle. The provision of both detectors allows more flexibility in choosing measurement conditions. Particles can be dispersed in a variety of liquids. Only liquid refractive index and viscosity needs to be known for interpreting the measurement results.

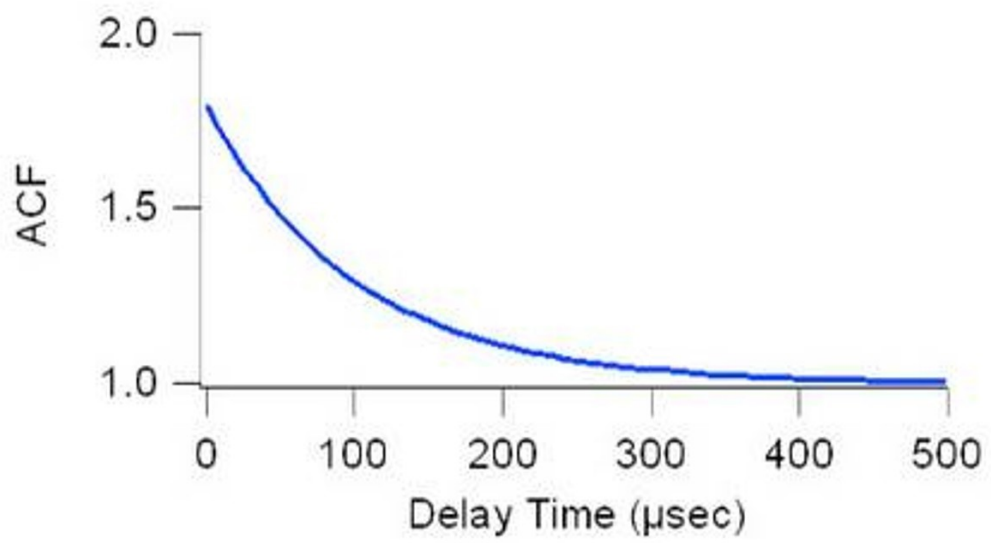
The obtained optical signal shows random changes due to the randomly changing relative position of the particles. This is shown schematically in the graph below.



The variations in the signal arise due to the random Brownian motion of the particles. Treating this random signal is discussed in the next section on extracting particle motion.

12.2 Extracting Particle Diffusion Coefficient: Dynamic Light Scattering Data Interpretation

The signal can be interpreted in terms of an autocorrelation function. Incoming data is processed in real time with a digital signal processing device known as a correlator and the autocorrelation function as a function of delay time, τ, is extracted.



$$C = \exp(-2\Gamma\tau)$$

For a sample where all of the particles are the same size, the baseline subtracted autocorrelation function, C, is simply an exponential decay of the following form:

Γ is readily derived from experimental data by a curve fit. The diffusion coefficient is obtained from the relation $\Gamma=D_t q^2$ where q is the scattering vector, given by $q=(4\pi n/\lambda)\sin(\theta/2)$. The refractive index of the liquid is n. The wavelength of the laser light is λ, and scattering angle, θ. Inserting D_t into the Stokes-Einstein equation above and solving for particle size is the final step

12.3 Analyzing Real Particle Size Distributions I: The Method of Cumulants and Z-average

The discussion above can be extended to real nanoparticle samples that contain a distribution of particle sizes. The exponential decay is rewritten as a power series:

$$C = \exp(-2\bar{\Gamma}\tau + \mu_2\tau^2 - \cdots)$$

Once again, a decay constant is extracted and interpreted to obtain particle size. However, in this case, the obtained particle size, known as the z-average size, is a weighted mean size. Unfortunately, the weighting is somewhat convoluted. Recall that the decay constant is proportional to the diffusion coefficient. So, by dynamic light scattering one has determined the intensity weighted diffusion coefficient. The diffusion coefficient is inversely proportional to size. So, in truth, the "z-average size" is the intensity weighted harmonic mean size. This definition differs substantially from that of the z-average radius of gyration encountered in the light scattering study of polymers.

Despite the convoluted meaning, the z-average size increases as the particle size increases. And, it is extremely easy to measure reliably. For these reasons, the z-average size has become the accepted norm for particle sizing by dynamic light scattering.

12.4 Analyzing Real Particle Size Distributions II: Size Distribution Data

While a detailed discussion is beyond the scope of this work, it is possible to extract size distribution data from DLS data. One can convert the measured autocorrelation function into what is known as an electric field autocorrelation function, $g1(\tau)$. Then use the following relationship between $g1(\tau)$ and the scattered intensity, S, for each possible decay constant, Γ. The overall electric field autocorrelation function is the intensity weighted sum of the decays due to every particle in the system.

Inversion of this equation, that is using experimentally determined values of g1(τ) to find values of S(Γ), will lead to information about the size distribution. Unlike the cumulants analysis discussed above, this is an ill-posed mathematical problem. Even so, the technique remains useful for interpreting DLS data.

$$g_1(\tau) = \int S(\Gamma) \exp(-\Gamma \tau) d\Gamma$$

12.5 comments

The underlying theory of measurement by dynamic light scattering was discussed. Many of the points on this web page are starting points for further investigation depending on the reader's analytic needs and interests. All of these equations and the analysis are handled automatically in the **HORIBA software**. As such, dynamic light scattering has found application for determining protein size, nanoparticle size, and colloid size.

https://www.horiba.com/en_en/en-en/technology/measurement-and-control-techniques/material-characterization/dynamic-light-scattering/

9.Extra data for reading only

12.6 https://www.biorxiv.org/content/10.1101/2020.10.15.341537v1.full

12.7 Materials and methods

12.7.1 Animals

Female BALB/c mice (6–8 weeks old) were obtained from Charles River and randomly assigned into cages in groups of 10 animals. The mice were allowed free access to water and rodent diet (Harlan, Israel). All animal experiments were conducted in accordance with the guideline of the Israel Institute for Biological Research (IIBR) animal experiments committee. Protocol numbers: #M-60-19, #M-30-20.

12.7.2 Production of SARS-CoV-2 antigens for immunization and *in vitro* assays

Recombinant SARS-CoV-2 spike glycoprotein, was expressed in pcDNA3.1^{+} plasmid, as recently described [26]. A stabilized soluble version of the spike protein (based on GenPept: QHD43416 ORF amino acids 1-1207) was designed to include proline substitutions at positions 986 and 987, and disruptive replacement of the furin cleavage site RRAR (residues at position 682-685) with GSAS. C-terminal his-tag as well as a strep-tag, were included in order to facilitate protein purification. Expression of the recombinant proteins was performed using ExpiCHO^{TM} Expression system (Thermoscientific, USA, Cat# A29133) following purification using HisTrap^{TM} (GE Healthcare, UK) and Strep-Tactin®XT (IBA, Germany). The purified protein was sterile-filtered and stored in PBS.

Human Fc-RBD fused protein was expressed using previously designed Fc-fused protein expression vector (Tal-Noy-Poral et al 2015), giving rise to a protein comprising of two RBD moieties (amino acids 331-524, see accession number of the S protein above) owing to the homodimeric human (gamma1) Fc domain (huFc). Expression of the recombinant proteins was performed using ExpiCHO^{TM} Expression system (Thermoscientific, USA) following purification using HiTrap Protein-A column (GE healthcare, UK). The purified protein was sterile-filtered and stored in PBS.

12.7.3 mRNA

CleanCap® firefly luciferase mRNA was a kind gift from BioNtech RNA Pharmaceuticals (Mainz, Germany). CleanCap®, pseudouridine-substituted Fc-

conjugated RBD mRNA (331-524 aa) was purchased from TriLink Bio Technologies (San Diego, CA, USA). The Fc-conjugated RBD mRNA was designed to include the exact translated Fc-RBD protein sequence as the recombinant protein.

Note https://www.trilinkbiotech.com/cleancap-fluc-mrna-5mou.html

12.8 LNP preparation and characterization

LNPs were synthesized by mixing one volume of lipid mixture of ionizable lipid, DSPC, Cholesterol and DMG-PEG (40:10.5:47.5:2 mol ratio) in ethanol and three volumes of mRNA (1:16 w/w mRNA to lipid) in acetate buffer. Lipids and mRNA were injected in to a micro fluidic mixing device Nanoassemblr® (Precision Nanosystems, Vancouver BC) at a combined flow rate of 12 mL/min. The resultant mixture was dialyzed against phosphate buffered saline (PBS) (pH 7.4) for 16 h to remove ethanol.

Particles in PBS were analyzed for size and uniformity by dynamic light scattering (DLS). Zeta potential was determined using the Malvern zeta-sizer (Malvern Instruments Ltd., Worcestershire, UK). RNA encapsulation in LNPs was calculated according to Quant-iT RiboGreen RNA Assay Kit (Thermo Fisher, Waltham, MA, USA).

12.9 Animal vaccination experiments

For *in vivo* LNP formulations screen, groups of 6-8-week-old female BALB/c mice were administered intramuscularly (50µl in both hind legs), intradermally (100µl) or subcutaneously (100µl) with luciferase mRNA (5µg) encapsulated with five different LNP formulations (LNPs #2, #5, #10, #14, #15). Luciferase expression was monitored as described in the bioluminescence imaging studies section. 28 days post-intramuscular injection, serum and spleen were collected from mice for evaluation of the immunologic response that developed towards luciferase.

For RBD mRNA vaccination studies, groups of 6-8-week-old female BALB/c mice were administered intramuscularly (50µl in both hind legs) or intradermally (100µl) with SARS-CoV-2 mRNA (5µg) encapsulated with LNP formulations #2 or #14.

For recombinant RBD (rRBD) vaccination studies, groups of 6-8-week-old female BALB/c mice were administered subcutaneously (100µl) with hFc-

rRBD (10μg), hFc-rRBD emulsified in complete/incomplete Freund's adjuvant (CFA/IFA), or adjuvant alone as control.

Both RBD mRNA-and recombinant RBD-immunized animals were boosted at day 25 with the same priming dose administered on day 0. Serum and spleens were collected on day 23 ("pre-boost") and 49 ("post-boost") for evaluation of immunologic response towards SARS-CoV-2 RBD, and measurement of cytokine secretion.

12.10 Bioluminescence Imaging Studies

Bioluminescence imaging was performed with an IVIS Spectrum imaging system (Caliper Life Sciences). Female BALB/c mice were administered D-luciferin (Regis Technologies) at a dose of 150 mg/kg intraperitoneally. Mice were anesthetized after receiving D-luciferin with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg) and placed on the imaging platform. Mice were imaged at 5 minutes post administration of D-luciferin using an exposure time of 60 seconds. Bioluminescence values were quantified by measuring photon flux (photons/second) in the region of interest using the Living IMAGE Software provided by Caliper.

12.11 ELISA

ELISA plates (Nunc) were pre-coated with recombinant luciferase (0.4μg/ml, Promega, #E1701) or spike protein (2μg/ml) overnight at 4°C in carbonate buffer. Plated were washed three times with PBST (PBS+0.05% Tween-20) and blocked with 2% BSA (Sigma-Aldrich, #A8022) in PBST for 1 h at 37 °C. After three washes with PBST, plates were incubated with serially diluted mouse sera for 1 h at 37 °C. Following washing, goat anti-mouse alkaline phosphatase-conjugated IgG (Jackson Immuno Research Labs, #115-055-003) was added for 1 h at 37 °C. The plates were washed with PBST and reactions were developed with p-nitrophenyl phosphate substrate (PNPP, Sigma-Aldrich, N2765). Plates were read at 405 nm absorbance and antibody titers were calculated as the highest serum dilution with an OD value above 2 times the average OD of the negative controls.

12.12 Cytokine assays

Splenocytes from immunized mice were incubated in the presence of SARS-CoV-2 spike protein (10μg/ml). Culture supernatants were harvested 48 h later and analyzed for cytokines by ELISA techniques with commercially available

kits. IL-2 (DY402), IL-4 (DY404) and IL-10 (DY417) kits were obtained from R & D Systems, Minneapolis, Minn.

12.13 Murine IFNγ ELISpot Assay

Mice spleens were dissociated in GentleMACS C-tubes (Miltenyi Biotec), filtered, treated with Red Blood Cell Lysing Buffer (Sigma-Aldrich, #R7757), and washed. Pellets were resuspended in 1ml of CTL-Test™ Medium (*CTL, #CTLT 005*) supplemented with 1% fresh glutamine, and 1 mM Pen/Strep (Biological Industries, Israel), and single cell suspensions were seeded into 96-well, high-protein-binding, PVDF filter plates at 400,000 cells/well. Mice were tested individually in duplicates by stimulation with recombinant luciferase (13µg/ml, Promega, #E1701), SARS-CoV-2 spike protein (10µg/ml), Concanavalin A (Sigma-Aldrich, #0412) (2µg/ml) as positive control, or CTL medium as negative control (no antigen). Cells were incubated with antigens for 24 h, and the frequency of IFNγ-secreting cells was determined using Murine IFNγ Single-Color Enzymatic ELISPOT kit (CTL, #MIFNG 1M/5) with strict adherence to the manufacturer's instructions. Spot forming units (SFU) were counted using an automated ELISpot counter (Cellular Technology Limited).

12.14 Plaque Reduction Neutralization Test (PRNT)

VSV-spike [27]stocks were prepared by infection of Vero E6 cells for several days. When viral cytopathic effect (CPE) was observed, media were collected, clarified by centrifugation, aliquoted and stored at −80°C. Titer of stock was determined by plaque assay using Vero E6 cells.

For plaque reduction neutralization test (PRNT), Vero E6 cells ($0.5*10^6$ cells/well in 12-well plates) were cultured in DMEM supplemented with 10% FCS, MEM non-essential amino acids, 2nM L-Glutamine, 100 Units/ml Penicillin, 0.1 mg/ml streptomycin and 12.5 Units/ml Nystatin (Biological Industries, Israel) overnight at 37°C, 5% CO_2.

Serum samples were 3-fold serially diluted (ranging from 1:50-1:12,500) in 400 µl of MEM supplemented with 2% FCS, MEM non-essential amino acids, 2nM L-Glutamine, 100 Units/ml Penicillin, 0.1 mg/ml streptomycin and 12.5 Units/ml Nystatin. 400 µl containing 300 PFU/ml of VSV-spike were then added to the diluted serum samples and the mixture was incubated at 37 °C, 5% CO_2 for 1 h. Monolayers were then washed once with DMEM w/o FBS and 200 µl of each serum-virus mixture was added in triplicates to the cells for 1 h. Virus mixture without serum was used as control. 1 ml overlay [MEM containing 2% FBS and 0.4% tragacanth (Sigma, Israel)] was added to each

well and plates were incubated at 37 °C, 5%CO_2 for 72 h. The number of plaques in each well was determined following media aspiration, cells fixation and staining with 1 ml of crystal violet (Biological Industries, Israel). NT50 was defined as serum dilution at which the plaque number was reduced by 50%, compared to plaque number of the control (in the absence of serum).

12.15 Transmission Electron Microscopy Analysis

A drop of aqueous solution containing LNPs was placed on the carbon-coated copper grid and dried. The morphology of LNPs was analyzed by a JEOL 1200 EX (Japan) transmission electron microscope

12.16 Statistical analysis

All values are presented as mean plus standard error of the mean (s.e.m). Antibody titers, neutralizing titers, ELISpot data and cytokine levels were compared using two-way ANOVAs or t-tests as depicted in the figure captions. All statistical analyses were performed using GraphPad Prism 8 statistical software.

https://www.precisionnanosystems.com/our-technology/reagents