# Supplementary Information

# 3D-Printed Microfluidic Device for High-Throughput Production of Lipid Nanoparticles Incorporating SARS-CoV-2 Spike Protein mRNA

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## Materials & Methods

### Preparation of Lipid Mixtures, mRNA, and LNPs

**Lipid mixture preparation.** Lipid mixtures in ethanol were prepared as previously described for the mRNA-1273 vaccine.[1] Briefly, heptadecan-9-yl-8-((2-hydroxyethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate (SM-102) (Ambeed Inc, United States of America), 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000) (Avanti Polar Lipids, United States of America), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) (Avanti Polar Lipids, United States of America), and cholesterol (Avanti Polar Lipids, United States of America) were dissolved in ethanol at 50, 1.5, 10, 38.5 mol%, respectively.

**Preparation of mRNA encoding SARS-CoV-2 spike protein.** To prepare mRNA molecules resembling those in mRNA vaccines against SARS-CoV-2, a plasmid insert was designed based on the sequence confirmed by comparative analyses of RNA extracted from original vials of Moderna mRNA-1273.[2] The plasmid insert sequence was designed with a BbsI restriction site for plasmid linearization prior to PCR amplification and a T7 promoter sequence to enable subsequent *in vitro* transcription (complete insert sequence shown in Figure S1). The plasmids (Twist Bioscience, United States of America) were linearized using BbsI restriction enzymes (New England Biolabs, United States of America) and then PCR amplified using 3' and 5' primers (Integrated DNA Technologies, United States of America) as shown in Figure S1. The PCR product was then transcribed using a MEGAscript T7 Transcription Kit (Invitrogen, United States of America) and the mRNA was purified using a MEGAclear Transcription Clean-up Kit (Invitrogen, United States of America). The sizes of the PCR product and transcription product were confirmed by agarose gel electrophoresis (Figure S2) using loading dyes and ladders obtained from New England Biolabs, United States of America. The resulting mRNA was expected to have 3996 bases. mRNA was stored at −20°C in 25 mM sodium acetate (pH 5) until use.

**Buffer exchange and sterilization of the LNPs.** LNPs collected from the device outlet were buffer exchanged to 20 mM Tris-Cl buffer (pH 7.5) using 50 kDa MWCO Amicon™ Ultra-0.5 Centrifugal Filter Units (MilliporeSigma™, United States of America) and then sterilized using 0.2 μm syringe filters (Pall Life Sciences, United States of America). Buffer-exchanged LNPs were stored at 4°C.

## Device Design and Fabrication

All microfluidic devices were designed using Autodesk Fusion 360 and fabricated using an SLA-DLP printer (Asiga MAX 27UV, Australia) with Printodent GR-10 resin (Pro3dure Medical, United States of America). The devices were 3D printed at a 10 μm layer thickness. The print duration for 4 devices was 12 hours, averaging a 3 hours/device print time. The resulting microchannels were washed with isopropanol at 1 mL/min for at least 30 minutes to remove residual uncured resin within the channels. The device exterior was smoothed to increase device transparency by applying a thin layer of resin between each surface of the device and a piece of cover glass, post-curing the device under a 36 Watt UV chamber (ProtoProducts, United States of America) for 30 minutes, and then removing the cover glass.

## LNP Characterization

**Size, Zeta Potential, and Particle Concentration.** LNP size, polydispersity index (PDI), and zeta potential were obtained from dynamic light scattering (DLS) (Malvern ZetaSizer Ultra, Malvern Panalytical, United Kingdom). Each data collection run was repeated at least 3 times; standard deviations across runs are reported in the Results & Discussion section below. Nanoparticle tracking analysis (NanoSight NS300, Malvern ZetaSizer Ultra, Malvern Panalytical, United Kingdom) was used for particle concentration measurements.

**mRNA Encapsulation Efficiency.** The mRNA encapsulation efficiency of the LNPs is defined as the fraction of mRNA encapsulated within the particles over the total mRNA in solution. This was calculated by measuring the fraction of unencapsulated mRNA over the total mRNA in a sample of LNPs:

$$\% \text{ Encapsulation Efficiency} = 1 - \frac{\text{unencapsulated mRNA}}{\text{total mRNA}} \times 100\%$$

Unencapsulated mRNA is free in solution while total mRNA can be freed by lysing the LNPs. The amount of mRNA was determined using RiboGreen fluorescence assay (Quant-it™ RiboGreen RNA Assay Kit, Invitrogen, United States of America) according to the manufacturer's protocol. The total mRNA in solution was determined by lysing the LNPs in the presence of 1% Triton™ X-100 (Thermo Scientific, Unites States of America) for 10 minutes at 37°C prior to binding. Fluorescence measurement was performed using BioTek Synergy H1 Microplate Reader (Agilent, United States of America). Each data point was obtained in duplicate.

**Cryo-TEM Imaging.** Based on the optimized parameters obtained as described below, LNPs were produced at FRR = 20, mRNA inlet concentration = 10 ng/μL, and mRNA inlet flow rate = 5 mL/min, and then concentrated 50 times using 50 kDa MWCO Amicon™ Ultra-0.5 Centrifugal Filter Units (MilliporeSigma™, United States of America). 8% sucrose was added to the samples as a cryoprotectant. In stained samples, 1% uranyl acetate (pH 4.0) (Ted Pella, Inc. United States of America) in water was mixed with the sample immediately before freezing. The sample was applied to glow-discharged C-flat™ holey carbon grids (CF-2/1-2Cu-50, Electron Microscopy Sciences, United States of America) at 22 °C and 100% relative humidity using an FEI Vitrobot Mark IV (ThermoScientific, United States of America). We first applied a 4 μL drop of sample to the grid, waited 10 minutes, and gently blotted the grid. Then, we applied another 4 μL drop of sample, waited 15 seconds, blotted the grid, and vitrified the sample into liquid ethane. The temperature of the sample was kept below -170°C at all times. The samples were imaged using a Talos™ F200C TEM (ThermoScientific, United States of America) operating at 200 kV. Images were acquired with a 4k × 4K Ceta CMOS camera at 36,000x magnification.

### COMSOL Multiphysics Flow Simulation

Ethanol concentration profiles in hydrodynamic flow focusing channels were simulated using COMSOL Multiphysics (version 5.6) with 3D channel geometries that are identical to the sheath flow region of the 3D-printed microfluidic devices (both 2-way and 4-way sheath configurations). The model assumes a no-slip boundary condition with $D = 1.6 \times 10^{-10}$ $m^2/s^3$ ethanol diffusivity in water. Computation was done with a mesh element size of 0.1 mm.

## Polydispersity of LNPs Generated after 1 Year

After 1 year of sustained usage, the OSEM device generated LNPs with a consistently low polydispersity index (PDI). The LNPs produced at a mRNA inlet flow rate of 4 mL/min and a flow rate ratio (FRR) of 20 exhibited an initial PDI of 0.045 ± 0.023. After repeated use over the course of a year, the same device produced LNPs with a consistently low PDI of 0.016 ± 0.016.

## Unloaded LNP Size in a 5-Hour High-Throughput Production Experiment

We conducted a 5-hour LNP production experiment at a total flow rate of 25.2 mL/min (FRR = 20, buffer inlet flow rate = 24 mL/min, and lipids inlet flow rate = 1.2 mL/min) without loading mRNA and measured the resulting LNP size using DLS. 20 mM Tris-HCl (pH=7) was used as an inlet buffer in place of the designated mRNA inlet. The LNP size of the first 50 mL collection had an average particle size of 82.78 nm with 17.84% polydispersity. The final 50 mL collection of the 5-hour run had an average particle size of 82.35 nm with 17.53% polydispersity. The LNP size did not change significantly after 5 hours of production using the same device, demonstrating stable production of the LNPs.

## Supplementary Figures

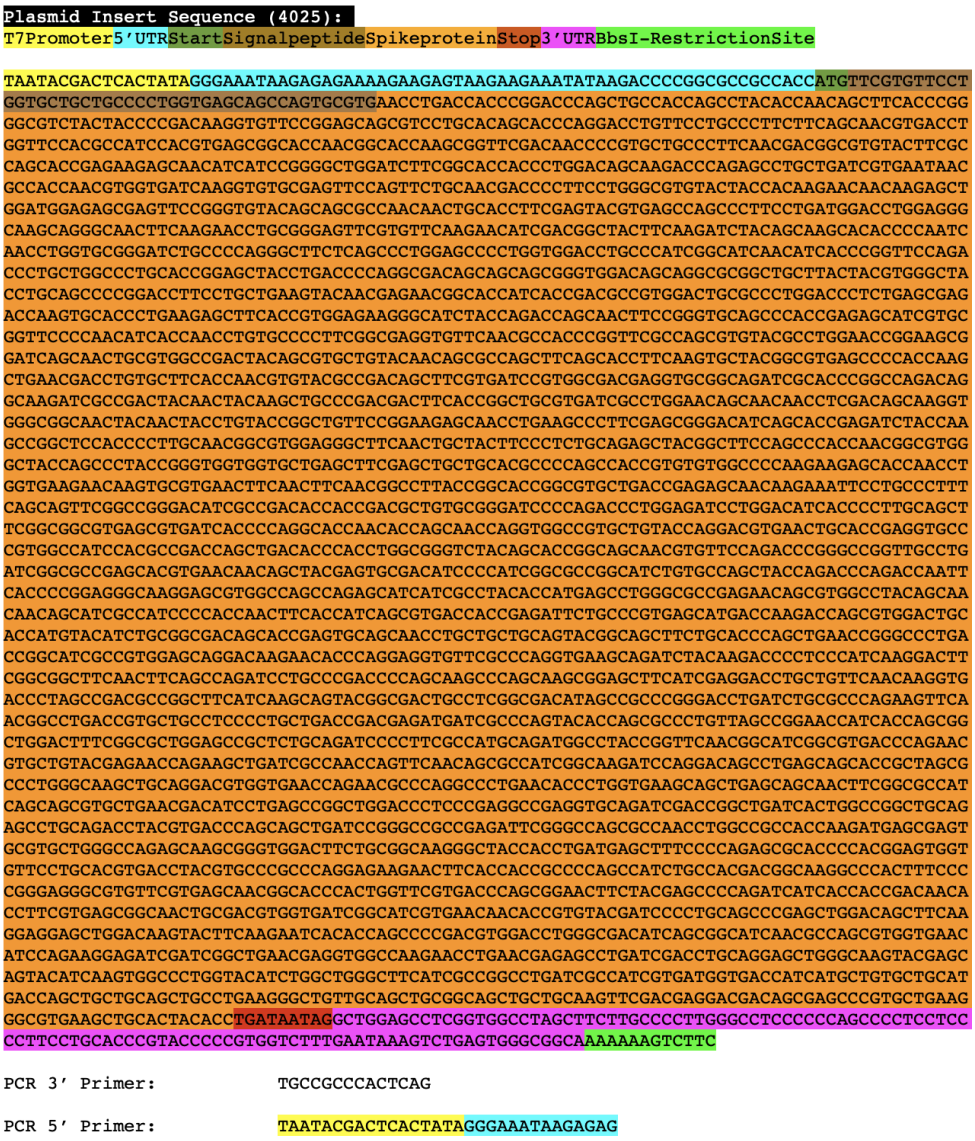


Figure S1: Plasmid insert sequence encoding SARS-CoV-2 spike protein and PCR primer sequences. Yellow: T7 promoter, blue: 3' untranslated region, hunter green: start codon, brown: signal peptide, orange: spike protein, red: stop codon, magenta: 3' untranslated region, light green: BbsI restriction site.

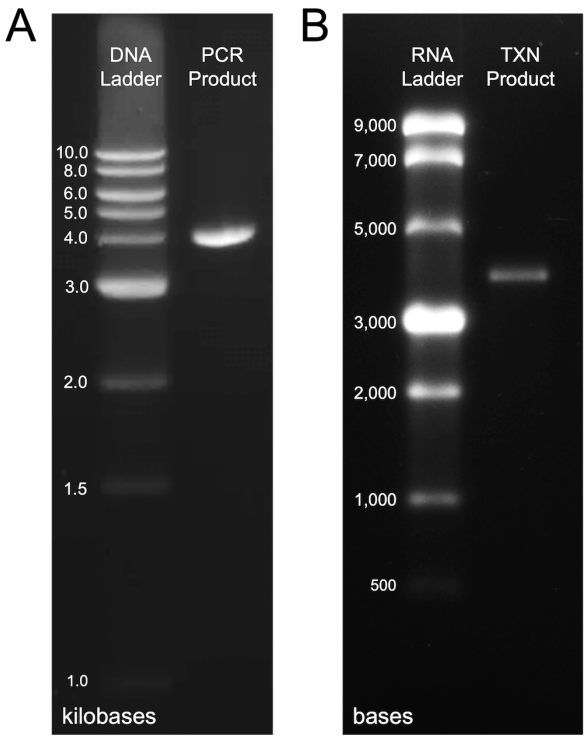


Figure S2: Gel electrophoresis results for **(A)** PCR and **(B)** transcription products from *p*DNA encoding SARS-CoV-2 spike protein.

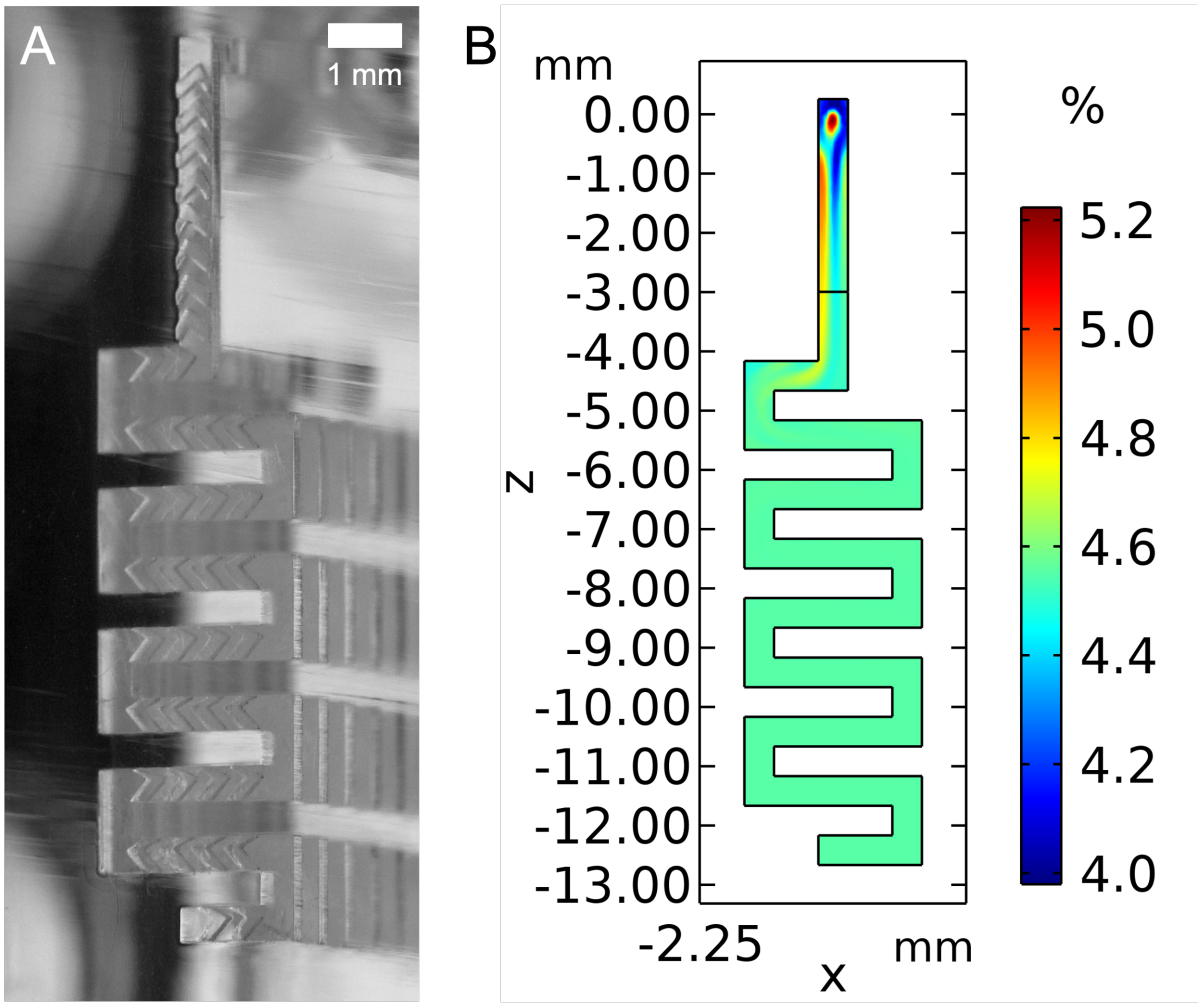


Figure S3: (A) Photo of the SHM region of the OSEM device. (B)COMSOL Multiphysics simulation results of ethanol/water mixing in the first zig-zag midplane of the SHM channel with 0.9 mixing index (FRR = 20 and water inlet flow rate = 5 mL/min).

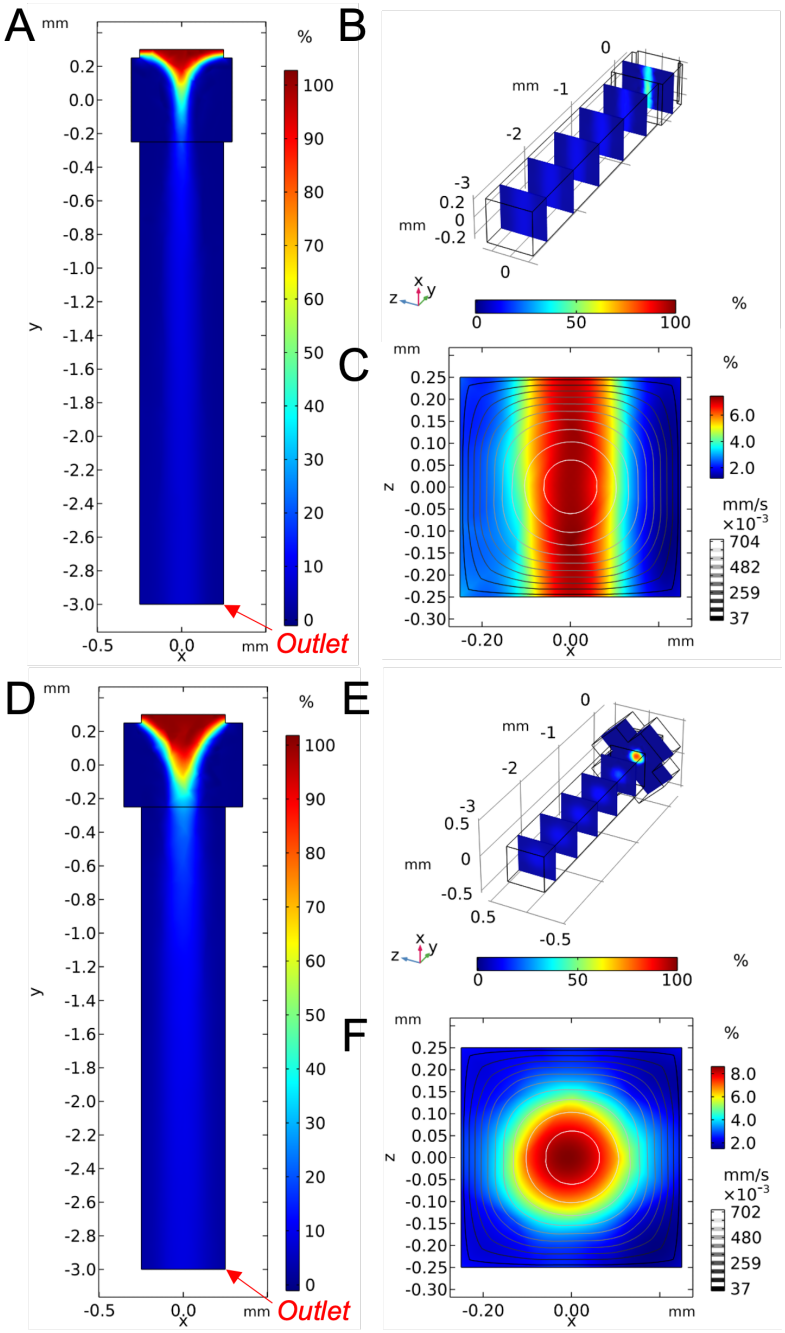


Figure S4: COMSOL Multiphysics simulation results showing ethanol/water hydrodynamic focusing at 5 µL/min total water inlet flow rate. The FRR was set to 20. **(A)** & **(D)** Results of a 2-way and a 4-way sheath flow regions of the device respectively, in the *x*-*y* plane. **(B)** & **(E)** Results for a 2-way and a 4-way sheath flow channel, respectively, in orthogonal view showing slices of the concentration profile in *x*-*z* planes. **(C)** & **(E)** Results for a 2-way and a 4-way sheath flow channel, respectively, in the *x*-*z* plane at outlet $y = -3$ mm with flow velocity contours.

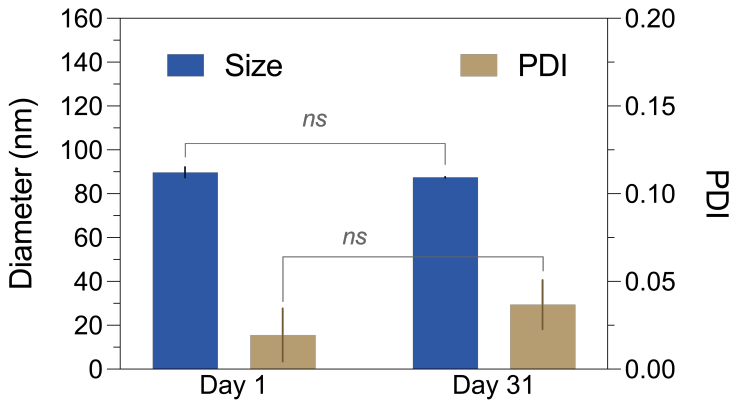


Figure S5: DLS size measurement results of LNPs on the day they were produced vs 30 days after storage at 4°C. The LNPs were produced at FRR = 20, inlet mRNA flow rate of 5 mL/min, and inlet mRNA concentration of 10 ng/µL. The size and PDI showed no significant statistical differences between measurements (Size: $p = 0.5048$; PDI: $p = 0.1816$). Error bars show standard deviations among > 3 repeated trials.

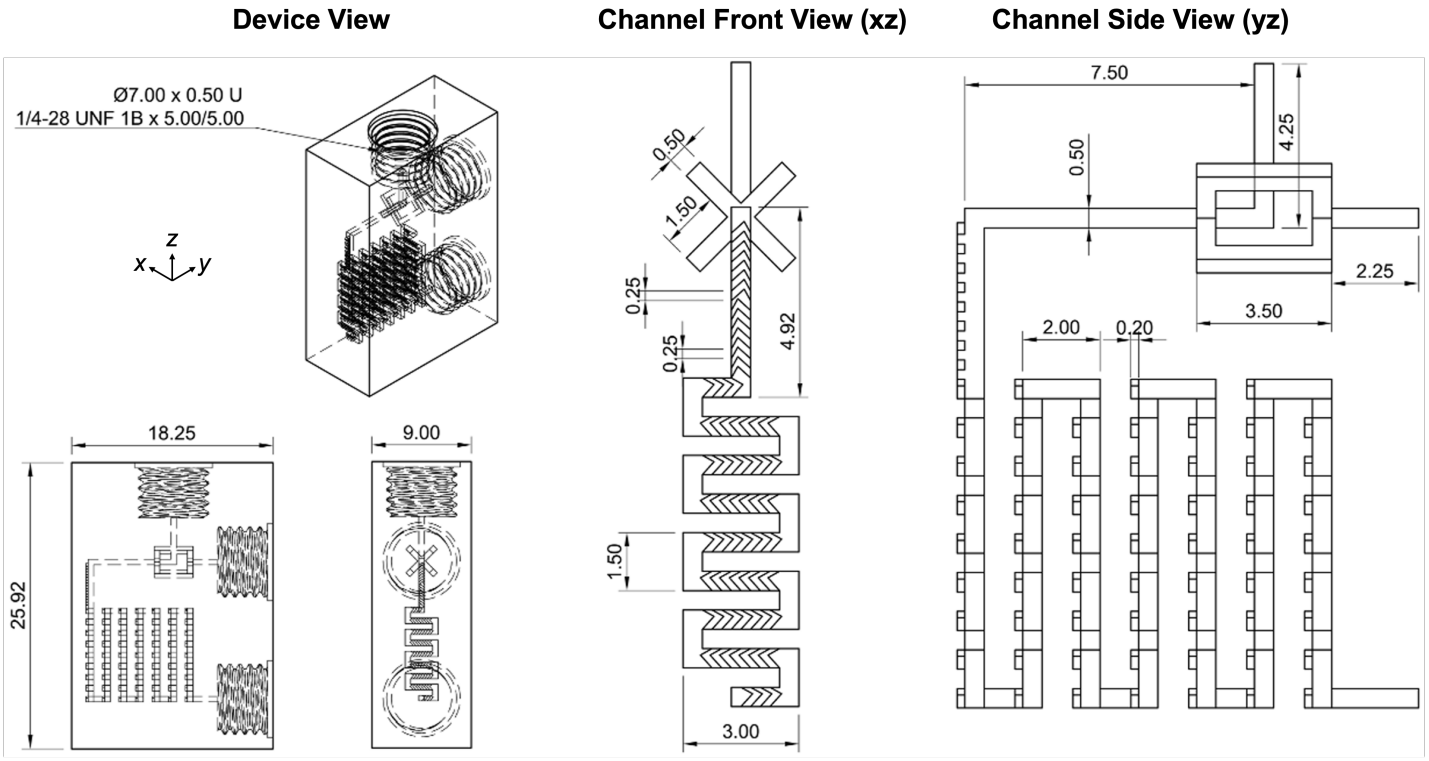


Figure S6: The OSEM design in drawings showing detailed design parameters. Unit: mm.

## References


(1) Schoenmaker, L.; Witzigmann, D.; Kulkarni, J. A.; Verbeke, R.; Kersten, G.; Jiskoot, W.; Crommelin, D. J. mRNA-lipid nanoparticle COVID-19 vaccines: Structure and stability. *International Journal of Pharmaceutics* **2021**, *601*, 120586.

(2) Jeong, D.-E.; McCoy, M.; Artiles, K.; Ilbay, O.; Fire, A.; Nadeau, K.; Park, H.; Betts, B.; Boyd, S.; Hoh, R.; Shoura, M. Assemblies of putative SARS-CoV2-spike-encoding mRNA sequences for vaccines BNT- 162b2 and mRNA-1273. **2021**.

(3) Hills, E. E.; Abraham, M. H.; Hersey, A.; Bevan, C. D. Diffusion coefficients in ethanol and in water at 298K: Linear free energy relationships. *Fluid Phase Equilibria* **2011**, *303*, 45–55.