Supplementary Information

Microfluidic Synthesis of Highly Potent Limit-Size Lipid Nanoparticles for In Vivo Delivery of siRNA

Nathan M. Belliveau, Jens Huft, Paulo J. C. Lin, Sam Chen, Alex K. K. Leung, Timothy J. Leaver, Andre W. Wild, Justin B. Lee, Robert J. Taylor, Ying K. Tam, Carl L. Hansen and Pieter R. Cullis

1.Mixing Performance of Microfluidic Staggered Herringbone Mixer (SHM)

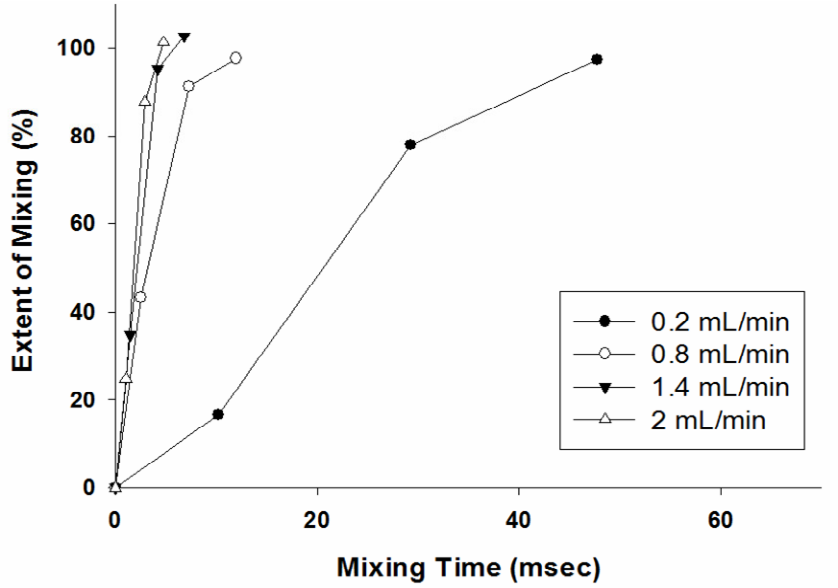
The mixing performance of the SHM was determined by mixing two fluorescein solutions and looking at the overall change in florescence in a manner similar to that described elsewhere[1]. One solution was fluorescent at pH 8.9 and the other non-fluorescent at pH 5.1. Both solutions contained 10 μM fluorescein and 0.5 M NaCl, while the pH 8.9 solution contained 14 mM phosphate and the pH 5.1 solution contained 1 mM phosphate. Images were collected along the channel length using an Olympus inverted confocal microscope using a 10x objective and Kalman filter mode with 2 scans per line. Twenty-five equally spaced slices were taken along the height of the channel and combined to determine total intensity profiles. For each position imaged, ten adjacent rows of pixels along the flow direction were averaged to obtain an intensity profile along the width of the channel and used to determine the extent of mixing[1].

$$Extent\ of\ Mixing = \frac{(I - I_0)}{(I_\infty - I_0)}$$

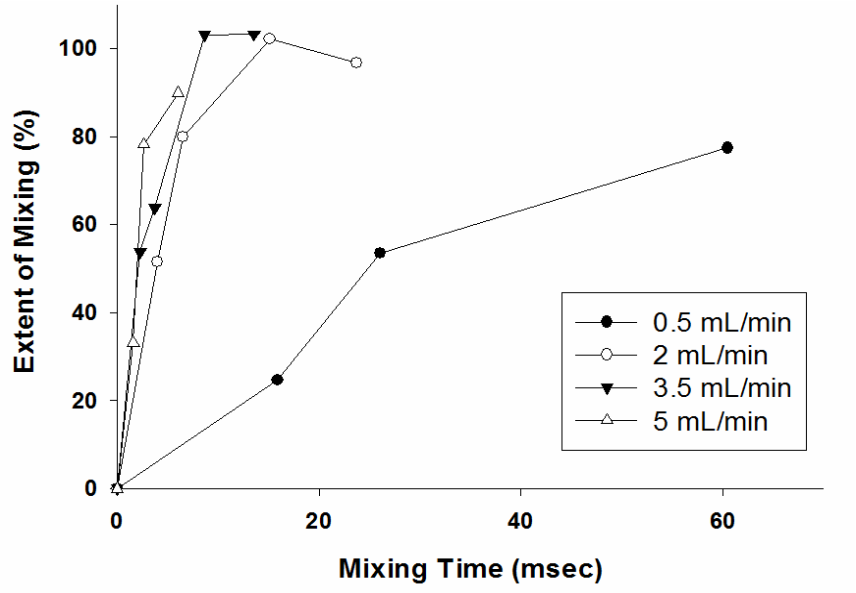
Where I is the average normalized intensity across the channel width, I_0 is the average normalized intensity at the inlet of the channel prior to mixing, and I_∞ is the average normalized

intensity when the fluids were completely mixed. The increase in fluorescence of the solution initially at pH 5.15 will overwhelm the small drop in fluorescence in the basic solution, resulting in an increase in total fluorescence intensity by a factor of two. The channel length required for mixing to occur (extent of mixing > 95%) was found to be between 0.8 cm and 1.0 cm and was relatively independent of flow rate. Two geometries were tested, the original SHM with a channel cross section of 79 µm x 200 µm, and one with a channel cross section of 130 µm x 300 µm (2.5 fold increase in cross sectional area). For the 79 µm x 200 µm SHM this resulted in mixing times of approximately 3 ms, 5 ms, 10 ms, and 45 ms for flow rates of 2 ml/min, 1.4 ml/min, 0.8 ml/min and 0.2 ml/min, respectively. For the 130 µm x 300 µm SHM this resulted in mixing times of approximately 5 ms, 8 ms, 12 ms, and greater than 45 ms for flow rates of 5 ml/min, 3.5 ml/min, 2 ml/min, and 0.5 ml/min, respectively.

a.

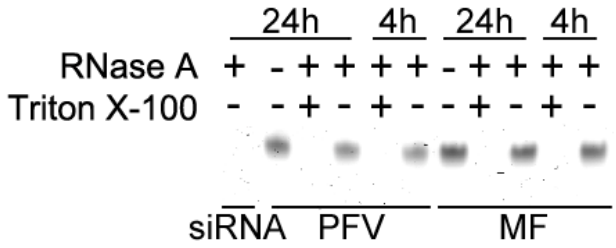


b.



Supplementary Figure S1: Influence of volumetric flow rate on extent of mixing in SHM. The extent of mixing was determined with two 10 µM fluorescein solutions, one fluorescent at pH 8.9 and the other non-fluorescent at pH 5.1, at approximately 2.1 mm, 6.2 mm, and 10.1 mm along the channel length. a. SHM with a channel cross-section of 79 µm x 200 µm. The two solutions were mixed at a 1:1 ratio using total flow rates of 0.2 ml/min (●), 0.8 ml/min (○), 1.4 ml/min (▼) and 2.0 ml/min (Δ). b. SHM with a channel cross-section of 130 µm x 300 µm. The two solutions were mixed at a 1:1 ratio using total flow rates of 0.5 ml/min (●), 2 ml/min (○), 3.5 ml/min (▼) and 5 ml/min (Δ). Note that only 5 equally spaced slices where taken when imaging the 130 µm x 300 µm geometry.

2.siRNA Protection Assay



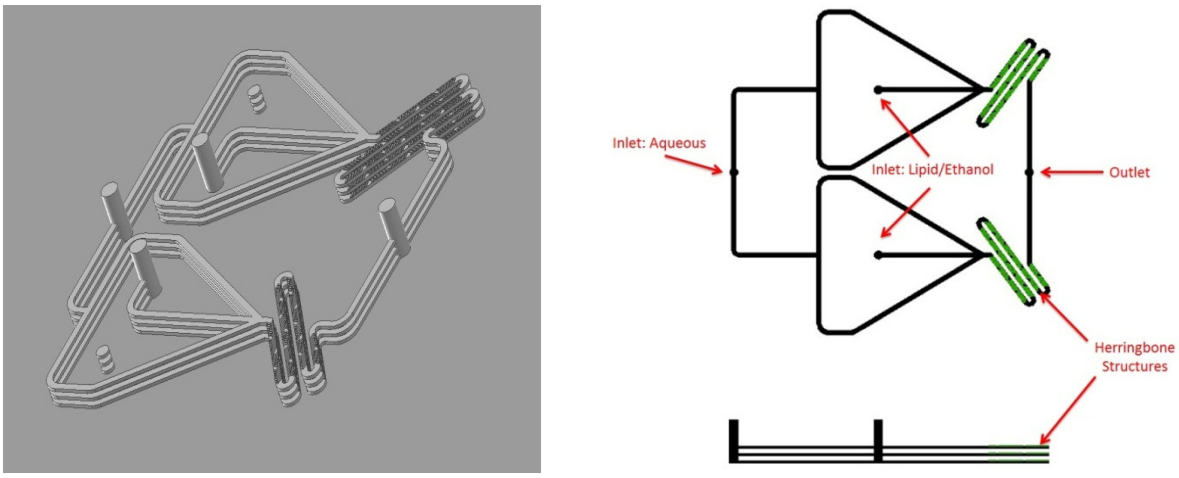
Supplementary Figure S2: Protection of siRNA from degradation during incubation in serum. 2.5ug of free or LNP encapsulated siRNA by either MF or PFV methods were incubated in mouse serum in the presence or absence of 0.5% Triton X-100 and 2.5ng/uL RNase A (Ambion). Reactions were quenched with deionized formamide after 1, 2, 4, and 24 hours and resolved on a 12% denaturing urea-polyacrylamide gel. Gels were stained with SYBR Safe (Invitrogen) in TBE buffer for 30 minutes and visualized on a Typhoon imager (GE Healthcare).

3.Microfluidic mixing using the SHM can be readily scaled by parallelization of mixers

Scale-up of a single microfluidic mixer was achieved by parallelizing six mixing elements. Two mixers were placed horizontally in parallel, with each mixer having one independent inlet, and one shared inlet, to create a 2x mixing element. Three of these 2x elements were then stacked vertically, to create a 6x mixer. The inlet line connecting the three vertical layers together is of negligible resistance compared to the mixing channels; therefore the three layers are effectively in parallel. Each of the six mixing elements was identical with a cross section of 130 um x 300 um. These dimensions were chosen to lessen the resistance of the channels and thus allow higher

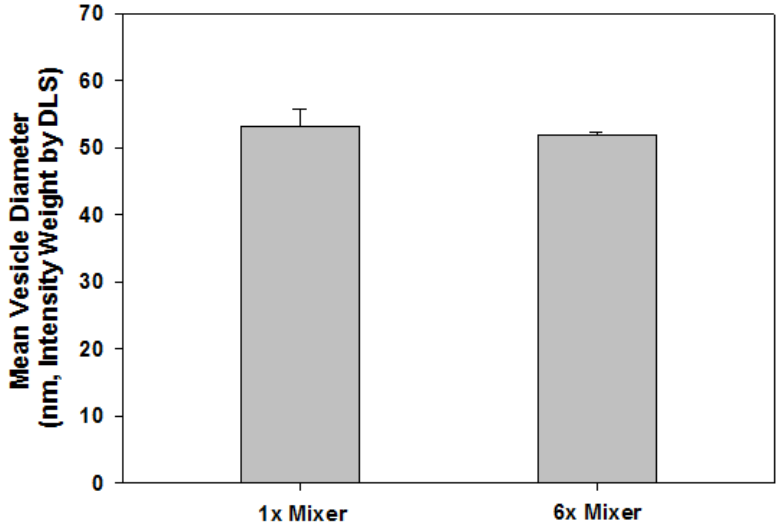
flow rates at lower pressures. Fluorescence imaging was used to verify that the mixing time for these larger cross section mixers was similar to those used in the main part of this study.

a. **b.**



Supplementary Figure S3: Schematic of 6x scale-up microfluidic device for formulation of lipid nanoparticles using individual microfluidic SHM mixers. a. CAD drawing of 6x scale-up SHM. b. Schematic of 6x scale-up SHM.

During formulation, a 20 ml syringe was used to feed the shared lipid-ethanol phase, while a 10 ml syringe was used for each of the two aqueous phase inlets. The lipid-ethanol syringe was driven by one syringe pump, while the two aqueous phase syringes were both driven by a second pump. Limit size LNP consisting of POPC/Cholesterol were generated using a 1x and 6x mixer resulting in essentially identical limit size particles of 53 nm diameter. Further parallelization in both the horizontal and vertical directions would allow further scaling-up.



Supplementary Figure S4: Scale-up of 1-palmitoyl, 2-oleoyl PC (POPC)/ Cholesterol Vesicles by parallelization of individual microfluidic SHM mixers. Limit size LNP composed of 1-palmitoyl, 2-oleoyl phosphatidylcholine (POPC)/cholesterol (55/45; mol/mol) were made by mixing lipid in ethanol with aqueous buffer at a buffer-to-ethanol volumetric flow rate ratio of 3:1, with a total flow rate of 12 ml/min in a single microfluidic mixer having a channel cross-section of 130 µm x 300 µm. The final lipid concentration after mixing was 8 mg/ml. Error bars represent standard deviation of multiple formulations made with the microfluidic mixers (*n*= 7 for single mixer and *n*= 3 for 6x mixer).

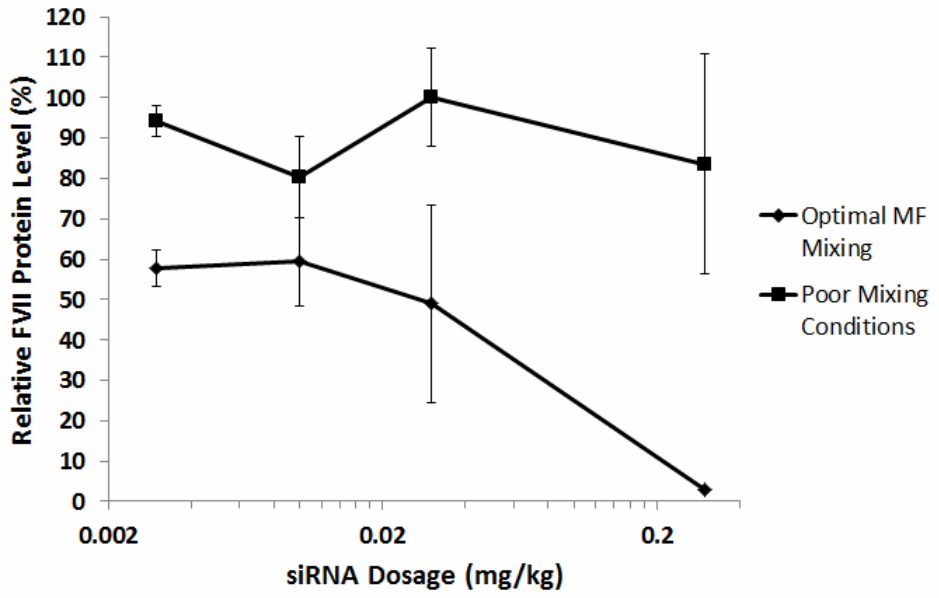
4.CryoTEM sample details

Table S1: LNP Size as determined by DLS and CryoTEM imaging

DLS		CryoTEM	
Mean Diameter	PDI	Mean Diameter	PDI
Sample 1 containing 1 mol% PEG-c-DMA			
55.4 nm	0.02	44.5 nm	0.023
Sample 2 containing 5 mol% PEG-c-DMA			
31.1 nm	0.05	22.4 nm	0.031

Sample 1 LNP composition: Dlin-KC2-DMA/DSPC/Cholesterol/PEG-c-DMA; 40/11.5/47.5/1; siRNA-to-total lipid ratio of 0.06 wt/wt. *Sample 2 LNP composition*: Dlin-KC2-DMA/DSPC/Cholesterol/PEG-c-DMA; 40/11.5/43.5/5; siRNA-to-total lipid ratio of 0.06 wt/wt. The LNP were produced at an initial lipid concentration of 30 mM in the lipid-ethanol phase prior to mixing with 25 mM acetate buffer, pH 4, containing siRNA. Formulation was performed at a 4 ml/min total flow rate with a siRNA-buffer:lipid-ethanol volumetric flow rate ratio of 3:1. CryoTEM size details determined from measurement of 120 individual LNP.

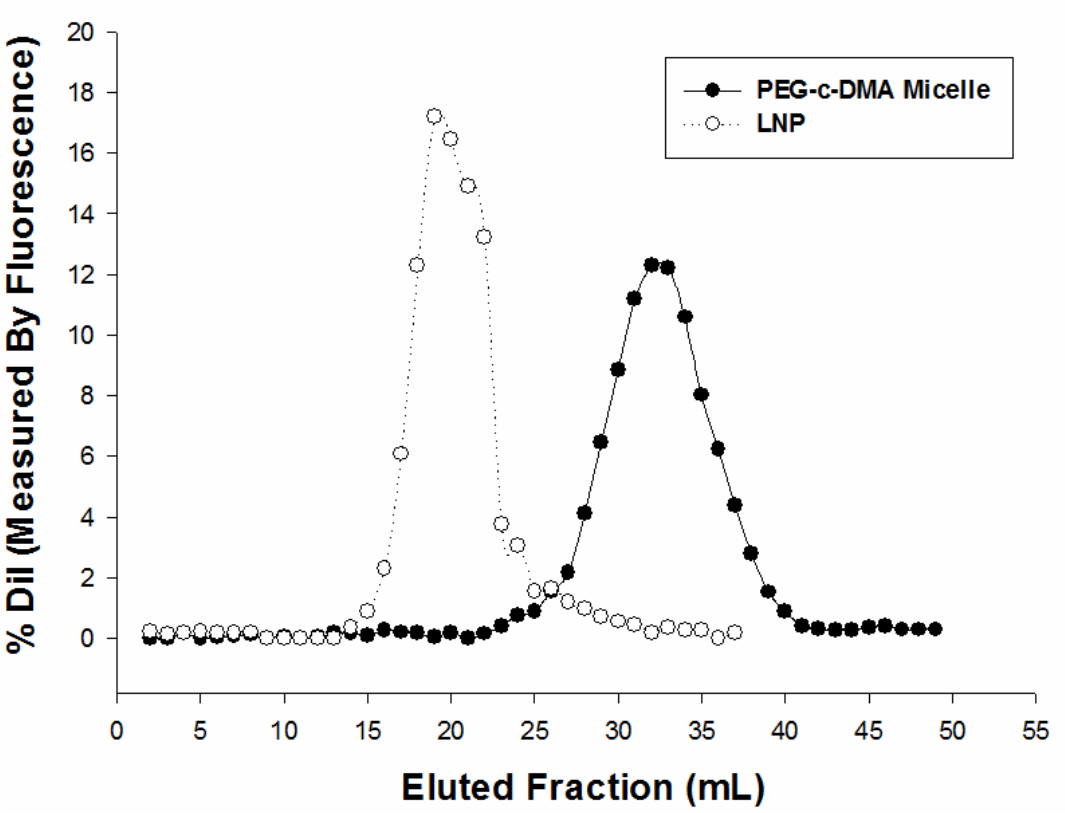
5.Comparison of LNP efficacy in FVII mouse model when produced under poor mixing conditions (larger, increased polydispersity LNP)



Supplementary Figure S5: Impact of formulation conditions and resulting LNP size on FVII gene silencing in mice. Formulation of LNP contained DLin-KC2-DMA/ DSPC/ Cholesterol/ PEG-c-DMA at a molar ratio of 60/9.75/29.25/1. Sample LNP labeled 'Optimal MF Mixing' (●) were produced under fast mixing (4 ml/min) at a volumetric flow rate ratio of 3:1 (aq:EtOH) and were 60.0 nm in diameter. Lipid concentration was 35 mM in ethanol. Sample

LNP labeled 'Poor Mixing conditions' (□) were produced at a 20 fold slower flow rate (0.2 ml/min) and at a higher lipid concentration (40 mM total lipid in ethanol). LNP were measured to be 170 nm in diameter. LNP siRNA-to-lipid ratio was maintained at 0.06 wt/wt. LNP diameter was determined by number-weighted mean diameter provided by DLS. Systemic injection of LNP-siRNA to mice was performed by tail vein injection (n=3 per dose level). Blood collection was performed after 24 hrs post-injection and factor VII levels were determined by colorimetric assay.

6.Separation of LNP containing 5 mol% PEG-lipid from potential PEG-c-DMA micelles using size exclusion chromatography



Supplementary Figure S6: Elution profiles of LNP containing 5 mol% PEG-c-DMA and PEG-c-DMA micelles. The LNP was composed of DLinKC2-DMA/DSPC/ Cholesterol/ PEG-c-DMA/ DiIc18 at a molar ratio of 40/11.5/43.3/5/0.2. Both LNP and micelles were produced under fast mixing (4 ml/min) at a volumetric flow rate ratio of 3:1 (aq:EtOH) and a lipid concentration of 30 mM in ethanol. LNP and micelle samples were run down a Sepharose CL-4B size exclusion column (28 cm x 1.5 cm) and collected into 1 ml fractions. 50 μL of each fraction was diluted with 150 μL methanol and fluorescence intensity measured in black 96 well plates (DiIC18, Life technologies, Ex. 549 nm, Em. 565 nm). Particle size of LNP was determined to be 25.8 nm (PDI 0.067, number weighting) prior to column separation. Following elution of the LNP sample, fractions 14 – 21 were combined, concentrated (Amicon Ultra-4 centrifugal units, Millipore) and measured to be 20.6 nm (PDI 0.11, number weighting).

Supplemental References

(1)Munson, M. S. ; Yager. P. *Analytica Chimica Acta.* **2004,** 501 (1), 63-71.