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Rapid Discovery of Potent siRNA-Containing Lipid Nanoparticles Enabled by Controlled Microfluidic Formulation

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Supporting Information

ABSTRACT: The discovery of potent new materials for in vivo delivery of nucleic acids depends upon successful formulation of the active molecules into a dosage form suitable for the physiological environment. Because of the inefficiencies of current formulation methods, materials are usually first evaluated for in vitro delivery efficacy as simple ionic complexes with the nucleic acids (lipoplexes). The predictive value of such assays, however, has never been systematically studied. Here, for the first time, by developing a microfluidic method that allowed the rapid preparation of high-quality siRNA-containing lipid nanoparticles (LNPs) for a large number of materials, we have shown that gene silencing assays employing lipoplexes result in a high rate of false negatives (~90%) that can largely be avoided through formulation. Seven novel materials with in vivo gene silencing potencies of >90% at a dose of 1.0 mg/kg in mice were discovered. This method will facilitate the discovery of next-generation reagents for LNP-mediated nucleic acid delivery.

The delivery of small interfering RNA (siRNA) into cells can induce silencing of target gene expression in a sequence-specific manner.[1,2] A key challenge in realizing its therapeutic potential is the development of safe and effective delivery vehicles for human use.[2] Lipids and lipid-like materials are promising reagents that have demonstrated delivery efficacy in vitro and in vivo.[2−7] The rational design of these reagents has proven difficult, despite recent advances.[8] High-throughput synthesis and screening is an excellent approach for discovering new materials with high delivery efficacy.[4,5,9] To improve the delivery efficacy in vivo, these reagents need to be formulated into lipid nanoparticles (LNPs) encapsulating siRNA, typically incorporating additional excipients such as cholesterol, poly(ethylene glycol)-conjugated lipids, and phospholipids to obtain desired biophysical and pharmacokinetic attributes.[6,7,10−12] Although many methods have been developed,[11,13,14] the formulation of a large number of high-quality siRNA-containing LNPs (siRNA-LNPs) remains a bottleneck in the screening process. Extrusion is widely used to control the size of LNPs prepared via other methods.[15] However, the formulation throughput is severely limited by the multistep, labor-intensive nature of extrusion and its susceptibility to membrane clogging for some formulations.[16] Moreover, when used to encapsulate nucleic acids,[12] the method requires milliliters of solution and can lead to significant loss of the expensive nucleic acids.[11] LNPs can also be formed by sonication, but the harsh technique can damage nucleic acids. Stepwise mixing of an ethanolic lipid solution and an aqueous solution of nucleic acids has been developed to prepare homogeneous NPs in one simple step;[10] the main hurdle to its application in high-throughput screening is the high cost of the tens of milliliters of nucleic acid solution[10,17] required by the original method for rapid mixing. Microfluidic hydrodynamic focusing has been used to control the size of empty lipid vesicles[18−20] and polymeric NPs,[21−23] but the method results in excessive dilution of particles and buildup of precipitate,[21] which can cause failure of the device. In the two reports where this method was used to prepare nucleic acid-containing LNPs, sonication and repeated dialysis were also required, and the particles still displayed considerable heterogeneity in size.[24,25] A formulation method matching the demands of high-throughput screening is urgently needed.

Here we report a formulation method based on stepwise ethanol dilution[10] that can produce a large number of siRNA-LNPs on a microliter scale. An alcoholic solution of the cationic lipid and other excipients is first rapidly mixed with an equal volume of aqueous siRNA solution. Water decreases the solubility of the lipid and promotes its self-assembly into LNPs, entrapping negatively charged siRNA through charge−charge interactions. The freshly formed siRNA-LNPs are further diluted with aqueous buffer to reduce the ethanol content and prevent aggregation of particles (Figure 1A). We performed both mixing steps in a microfluidic channel fabricated with polydimethylsiloxane using soft lithography[26] (Figure 1B). Periodic trenches and ridges were asymmetrically arranged on the floor of the channel [Figure 1C and Figure S1 in the Supporting Information (SI)] to promote chaotic mixing of the solutions as previously reported.[27] Rapid mixing was confirmed by measuring the fluorescence from the diffusion-limited binding between Ca^{2+} and fluo-4.[28] Mixing was 90% complete within ~8 ms when both solutions flowed at 300 μL/min (Figure S2). As little as 10 μL of each solution can be reliably mixed in milliseconds. To facilitate parallel formulation, we

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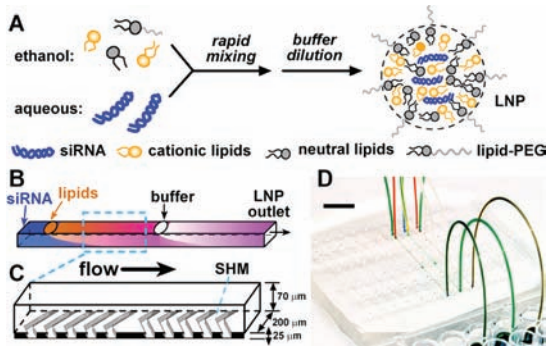

Figure 1. Formulation of siRNA-LNPs using rapid microfluidic mixing. (A) Schematic illustration of LNP formation through stepwise ethanol dilution. (B) Formation of siRNA-LNPs by mixing solutions in a microfluidic channel. (C) Schematic of the microfluidic channel with groove structures for rapid mixing. (D) Photograph of a microfluidic device with 24 channels for parallel formulation. Tubing filled with food coloring and buffer solution was connected to three of the channels to enhance visualization. Scale bar: 1.0 cm.

fabricated on each device 24 mixing channels (Figure 1D) that were used in subsequent experiments.

As an example of formulation using the device, we prepared siRNA-LNPs from C12-200, a lipid-like compound shown to deliver siRNA to the liver with high efficiency.[5] The compositions of the solutions used are provided in the SI. To test the effect of mixing on the particle size, we mixed solutions at different flow rates using the microfluidic device or manually by repeated pipetting. The effective diameters of the siRNA-LNPs, as determined by dynamic light scattering, were highly reproducible between repeated experiments and were found to decrease at higher flow rates and level off at ~70 nm for flow rates above 200 μL/min. Particles formed with pipet mixing had an effective diameter of ~180 nm (Figure 2A). The size distribution of siRNA-LNPs was further characterized using cryogenic transmission electron microscopy (cryo-TEM)

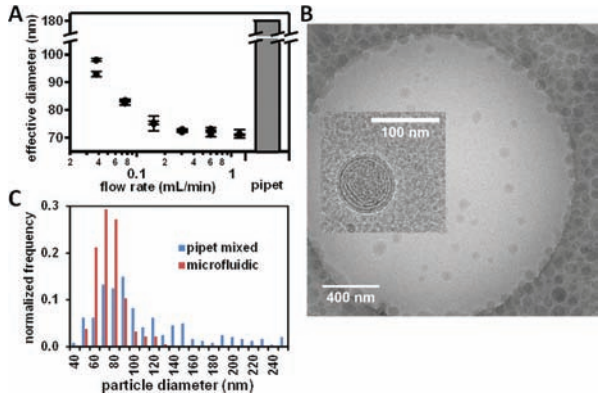

Figure 2. The size of siRNA-LNPs can be reproducibly controlled by microfluidic mixing. (A) Effective diameter of the siRNA-LNPs as a function of the mixing flow rate. For each flow rate, particle sizes of two independently prepared samples are shown. Error bars indicate the range of data from two DLS measurements. The effective diameter of LNPs prepared by pipet mixing of solutions (gray bar) is shown for comparison. (B) Cryo-TEM image of siRNA-LNPs prepared in the microfluidic device at 300 μL/min. Images for pipet-mixed particles are shown in Figure S4. (C) Microfluidic-formulated siRNA-LNPs had a narrower size distribution than pipet-mixed particles. Each histogram was built from counting 200−250 randomly selected particles.

(Figure 2B and Figure S4). Microfluidic LNPs (300 μL/min) were spheroid particles with a narrow distribution of diameters between 60 and 90 nm. The diameter of the pipet-mixed formulation was much more heterogeneous, with a large population of particles above 100 nm (Figure 2C). Radiation-sensitive ring structures consistent with nucleic acids in liposomes can be observed in the larger particles (Figure 2B and Figure S4). The efficiency of siRNA entrapment was determined using the RiboGreen fluorescence assay, which measures the amount of particle-entrapped siRNA as a percentage of the total siRNA.[29] The siRNA entrapment efficiency was ~80% under all conditions (Figure S3).

The dependence of particle size on the mixing flow rate is consistent with our understanding of the mixing behavior in the microfluidic mixer and with the theory of vesicle formation under nonequilibrium conditions.[30] According to this theory, LNP formation involves intermediate disklike planar fragments whose edges are stabilized by ethanol. During dilution of the ethanol, the planar fragments can grow by fusion. At even lower ethanol concentrations, the destabilized fragments bend to form closed NPs. When ethanol is diluted instantaneously to low concentrations, the planar fragments have little time to grow before closing into vesicles, resulting in smaller particles. It is known that the time required for mixing in the microfluidic mixer, t_m, decreases with flow velocity, U, as follows: $t_m \sim \lambda/[U \ln(Ul/D)]$, where λ and l are characteristic lengths determined by the geometry of the device and D is the diffusion coefficient.[27] At low flow rates, mixing is slow, and pockets of high ethanol concentration develop, favoring stabilization and growth of intermediate fragments that lead to larger LNPs. Our result is in qualitative agreement with previous studies on empty liposome formation using microfluidic hydrodynamic focusing.[19]

The fact that the particle diameter and encapsulation efficiency were constant at all flow rates above 200 μL/min led us to test whether the syringe pumps used to control the flow rates could be eliminated. Two 25 μL glass syringes, one filled with lipid solution and the other with siRNA solution, were fixed side by side on a plastic rack and connected to the microfluidic mixing device with tubing (Figure 3A). The outlet of the device was connected to an Eppendorf tube filled with 50 μL of phosphate-buffered saline for particle dilution. To generate siRNA-LNPs, we manually pushed the plungers of the two syringes simultaneously with an estimated flow rate higher than 300 μL/min. This method produced reproducible

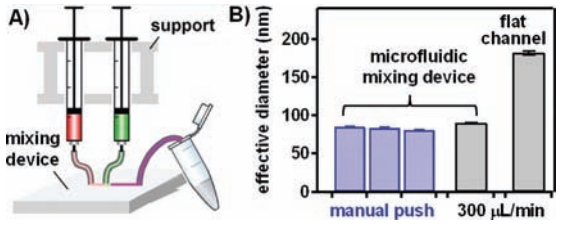

Figure 3. Preparation of siRNA-LNPs without the use of syringe pumps. (A) Schematic showing two syringes being simultaneously compressed to mix their contents in the microfluidic device. (B) Effective diameters of siRNA-LNPs prepared in microfluidic mixing devices without syringe pumps (blue bars), with syringe pumps (middle gray bar), and in a microfluidic channel without mixing features (right gray bar). Error bars indicate the range of data from two DLS measurements.

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particle sizes: the effective diameter ranged from 81.2 to 85.4 nm for three trials, compared with an effective diameter of 90.5 nm for particles made at a flow rate of 300 μL/min controlled by syringe pumps (Figure 3B). In contrast, ~180 nm particles were produced when the same solutions were passed through a microfluidic channel without the mixing features (flat channel). This "equipment-free" method of LNP formation decreases the expertise and capital that are required for traditional formulation methods and could therefore be a useful tool for facilitating LNP research.

Microfluidic formulation allows the preparation of well-defined LNPs that can be used to evaluate many materials for siRNA delivery both in vitro and in vivo. We applied this method to formulate a pilot library of lipid-like materials (cationic lipids)[5] into LNPs for siRNA delivery in cell culture and in mice. A total of 70 compounds (Figure 4A,B) were

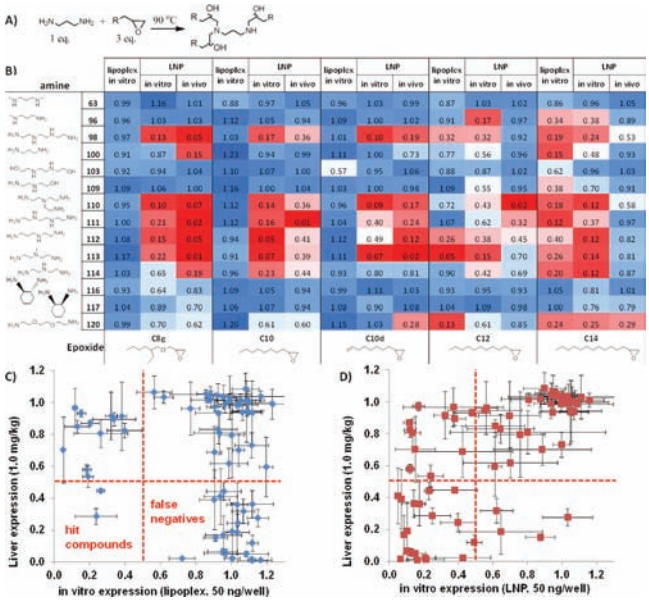

Figure 4. LNP formulation improves the screening for siRNA delivery reagents. (A) Reaction used to synthesize the pilot library of lipidoids for siRNA delivery. The materials were tested for in vitro and in vivo siRNA delivery as simple lipoplexes or LNPs. (B) Heat map of remaining gene expression (normalized against negative controls) in vitro and in vivo. siRNA for luciferase and factor VII were used for in vitro (n = 4) and in vivo (n = 3) experiments, respectively. Error bars are given in Figure S5. (C) Plot showing the correlation between in vitro (lipoplex) and in vivo gene expression. (D) Correlation between in vitro (LNP) and in vivo gene expression.

formulated. The in vitro LNPs encapsulated siRNA targeting firefly luciferase expression in dual-glow HeLa cells, and the delivery efficacy was evaluated by measuring the luciferase activity.[4] The in vivo LNPs encapsulated siRNA targeting factor VII expression in mouse liver.[5] Each in vivo siRNA-LNP formulation was prepared with a total volume of 1.0 mL and a final siRNA concentration of ~0.15 mg/mL and injected into mice via tail vein at a dose of 1.0 mg/kg (total mass of siRNA/mice body weight). The delivery efficacy in mice was determined by measuring the reduction of factor VII protein in blood 48 or 72 h after injection. This experiment allowed us to test the extent to which the in vitro assays predict the delivery efficacy in vivo. For comparison, simple lipoplexes were also formed by mixing the lipid-like material with luciferase-siRNA in aqueous solution, and the silencing in dual-glow HeLa cells was measured as previously reported.[4,5,31,32]

The results indicated that using LNPs greatly improved the screening of siRNA delivery materials (Figure 4). Indeed, previous screening on this library was conducted using simple lipoplexes, and we had only one compound, C12-200, that silenced factor VII by >90% in vivo at the tested dose of 1.0 mg/kg. By rescreening part of this same library using LNPs, we discovered seven new compounds with comparable potency (Figure 4B). When hit compounds were defined as those reducing gene expression by more than 50%, the in vitro assay using lipoplexes correctly predicted only two of 23 in vivo hit compounds (9%) while falsely rejecting the other 21 (91%) (Figure 4C). The in vitro assay using LNPs identified 83% of the hit compounds (Figure 4D). Moreover, the LNP-based in vitro assay identified all of the compounds with >90% gene silencing in vivo as hit compounds, while the lipoplex-based assay falsely rejected all of them. The four "false negatives" of the LNP-based in vitro assay are also interesting, because they may be indicative of liver-specific delivery.[33]

Analysis of structure–function relationships suggests that the activity of the lipoplex is disproportionally dominated by the length of hydrocarbon chain in the cationic lipid. When tested as lipoplexes, only compounds with longer hydrocarbon chains (C12 and C14) were represented in the in vitro hits, while the LNP hits included hydrocarbon chains of all tested lengths (Figure 5A). We chose cationic lipids having four different

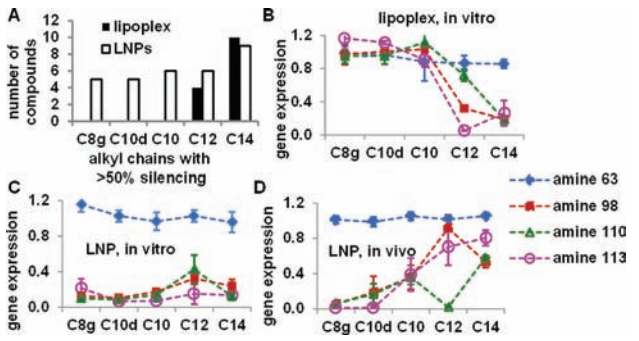

Figure 5. The siRNA delivery potencies of LNPs and lipoplexes show different dependences on the hydrocarbon chain length of the cationic lipid. (A) Number of in vitro hit cationic lipids with different hydrocarbon chain lengths when tested as LNPs (open bars) or lipoplexes (black bars). (B–D) Normalized remaining gene expression as a function of the hydrocarbon chain length for cationic lipids with four different amine headgroups. The opposite structure–function relationship between (B) and (D) should be noted. The original gene expression data are shown in Figure 4B. Error bars indicate ±1 standard deviation (B, C: n = 4; D: n = 3).

amine headgroups and plotted their siRNA delivery efficacies in vitro and in vivo as functions of the hydrocarbon chain length (Figure 5B–D). All of the tested cationic lipids with headgroup amine 63 were inactive. For the other three amine headgroups, the potency of the lipoplex increased with increasing hydrocarbon chain length, while the in vitro potency of the LNPs did not change significantly. The in vivo potency of LNPs, however, decreased with increasing hydrophobicity of the cationic lipids. This suggests that different interactions are responsible for the efficacies of lipoplexes and LNPs and that assays using lipoplexes are therefore poor indicators of LNP efficacy.

In conclusion, we have demonstrated improved screening of lipid-like materials for siRNA delivery using a small-scale

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microfluidic formulation method. Preparation of LNPs containing siRNA was shown as an example, but the method can be used to prepare polymer NPs and LNPs encapsulating other nucleic acids such as DNA with little modification (data not shown). The reproducible preparation of LNPs without using syringe pumps may also facilitate its adoption by nonspecialists of microfluidics, provided that the mixing devices are available. The method enabled us to test the correlation between LNP-mediated siRNA delivery in vitro and in vivo for a large number of materials. While the in vitro assay using LNPs predicted most of the effective compounds for in vivo delivery to the liver, the same in vitro assay using lipoplexes predicted only ~10% of the functional in vivo delivery materials and falsely rejected all of the reagents that were most potent in vivo. In view of the popularity of lipoplexes in previous in vitro assays,[4,5,31,32] attention should be paid to the possibility of false negatives. Even though in vivo assays of an entire library may not always be practical, we strongly recommend formulating cationic lipids into LNPs for all in vitro assays. The rapid and small-scale formulation method presented here may facilitate the discovery of better delivery reagents for polynucleotides.

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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