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Characterization of Lipid Nanoparticles Containing Ionizable Cationic Lipids Using Design-of-Experiments Approach

Takeshi Terada,* Jayesh A. Kulkarni, Ariel Huynh, Sam Chen, Roy van der Meel, Yuen Yi C. Tam, and Pieter R. Cullis

ABSTRACT: Lipid nanoparticles (LNPs) containing short-interfering RNA (LNP-siRNA systems) are a promising approach for silencing disease-causing genes in hepatocytes following intravenous administration. LNP-siRNA systems are generated by rapid mixing of lipids in ethanol with siRNA in aqueous buffer (pH 4.0) where the ionizable lipid is positively charged, followed by dialysis to remove ethanol and to raise the pH to 7.4. Ionizable cationic lipids are the critical excipient in LNP systems as they drive entrapment and intracellular delivery. A recent study on the formation of LNP-siRNA systems suggested that ionizable cationic lipids segregate from other lipid components upon charge neutralization to form an amorphous oil droplet in the core of LNPs. This leads to a decrease in intervesicle electrostatic repulsion, thereby engendering fusion of small vesicles to form final LNPs of increased size. In this study, we prepared LNP-siRNA systems containing four lipid components (hydrogenated soy phosphatidylcholine, cholesterol, PEG-lipid, and 1,2-dioleoyl-3-dimethylammonium propane) by microfluidic mixing. The effects of preparation parameters [lipid concentration, flow rate ratio (FRR), and total flow rate], dialysis process, and complex formation between siRNA and ionizable cationic lipids on the physicochemical properties [siRNA entrapment on the particle size and polydispersity index (PDI)] were investigated using a design of experiments approach. The results for the preparation parameters showed no impact on siRNA encapsulation, but lipid concentration and FRR significantly affected the particle size and PDI. In addition, the effect of FRR on the particle size was suppressed in the presence of anionic polymers such as siRNA as compared to the case of LNPs alone. More intriguingly, unlike empty LNPs, a decrease in the PDI and an increase in the particle size occurred after dialysis in the LNP-siRNA systems. Such changes by dialysis were suppressed at FRR = 1. These findings provide useful information to guide the development and manufacturing conditions for LNP-siRNA systems.

INTRODUCTION

Lipid nanoparticles (LNPs) containing ionizable cationic lipids are the most advanced nonviral nucleic acid delivery systems.1,2 The ionizable cationic lipid component of LNP-short-interfering RNA (LNP-siRNA) systems, with an apparent pKa of less than 7.0, plays a critical role in siRNA entrapment and endosomal escape within the cell and is also involved in dictating the internal morphology of the LNPs.3–5 An established rapid-mixing method for the preparation of LNP-siRNA systems is microfluidics mixing as it affords controlled mixing at the nanoliter scale.7–9 LNP-siRNA systems are generated by mixing an ethanolic solution of lipids with siRNA in aqueous buffer at pH 4.0 where ionizable cationic lipids are positively charged. Subsequently, LNP-siRNA suspensions are dialyzed to remove ethanol and to neutralize the pH, leading to LNPs with a relatively neutral surface.

A recent study showed that as the pH is raised to neutral, an increasing proportion of the ionizable cationic lipids adopts a neutral form, thus decreasing the intervesicle electrostatic repulsion, destabilizing the bilayer structure, and engendering vesicle fusion which leads to an increase in the LNP size.6,10,11 As the neutral ionizable lipids partition to the LNP core forming an oil-droplet phase, the particles fuse to compensate for the decreased surface-to-core lipid ratio. Changes of LNP-siRNA physicochemical properties (LNP size, surface composition, and morphology) have been shown to affect the in vivo potency.12–14 Therefore, understanding the effect of ionizable cationic lipids on the physicochemical properties of LNP-siRNA systems from a manufacturing perspective is important for robust process development. However, only limited reports are available on the impact of various formulation parameters on the physicochemical parameters of LNP systems.

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In this study, we investigated the effect of preparation parameters [lipid concentration, flow rate ratio (FRR), and total flow rate (TFR)] on the particle size, polydispersity index (PDI), and siRNA entrapment of LNPs prepared by microfluidics, including before and after dialysis against neutral solution [phosphate-buffered saline (PBS)]. Furthermore, the relationship between the preparation parameters and the properties was evaluated for LNPs with and without siRNA by performing the same procedure for empty LNPs. A design of experiments (DoE) approach was used to systematically evaluate these parameters, resulting in a decreased number of formulation runs. In order to implement a DoE approach, LNPs were prepared using hydrogenated soy phosphatidylcholine (HSPC), cholesterol (Chol), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000] (ammonium salt) (PEG-DSPE), which have the added benefit of being clinically approved lipid compounds. In addition, we used, 1,2-dioleoyl-3-dimethylammonium propane (DODAP) that has a reported apparent pKa of 6.58. Supporting Information studies were performed to provide insight on the mechanistic effect of manufacturing conditions and formulation on the physicochemical properties.

## MATERIALS AND METHODS

### Materials.
Lipid HSPC, PEG-DSPE, and DODAP were purchased from Avanti Polar Lipids (Alabaster, AL). Chol and hyaluronic acid (HA) sodium (MW: 8000–15,000) were purchased from Sigma-Aldrich (St. Louis, MO). The siRNA (siGAPDH) for this study was purchased from Integrated DNA Technologies (San Diego, CA). The siRNA sense and antisense strand sequences are mCmUrCmAr-UmUrCrGrUrGmUrAmCrArAr CrGrAmAT, rArUrCrGrUrGmUrCmArUmArCrCrArGrCrArArUm-GmGrU respectively.

### Preparation of LNPs/LNP-siRNA Systems.
LNPs were prepared as previously described. Briefly, lipid components composed of DODAP/Chol/HSPC/PEG-DSPE (50/10/39/1 mol %) were dissolved in ethanol to a concentration of 10–30 mM total lipid. The aqueous phase consisted of 25 mM sodium acetate pH 4 buffer. Since it has been reported that the pH of the aqueous phase affects the entrapment of nucleic acids in LNPs, the pH was adjusted to be less than 0.5%.

### Analysis of LNPs.
Particle size and PDI analysis of empty LNPs and LNP-siRNA systems were carried out using dynamic light scattering with a Malvern Zetasizer (Worcestershire, UK). In order to avoid the influence of ethanol on the measurement, the concentration of ethanol in the sample was adjusted to be less than 0.5%. Encapsulation efficiency of siRNA was determined using the Quant-iT Ribogreen RNA assay (Life Technologies, Burlington, ON). Briefly, LNP-siRNA was incubated at 37 °C for 10 min in the presence or absence of 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) followed by the addition of the ribogreen reagent. The fluorescence intensity (Ex/Em: 480/520 nm) was determined, and the samples treated with Triton X-100 represent total siRNA while untreated samples represent unencapsulated siRNA. Total lipid was determined by measuring the Chol content using the Chol E assay (Wako Chemicals, Richmond, VA), and the siRNA concentration was determined by measuring the absorbance at 260 nm.

### DOE Approach.
The experimental data were analyzed with the statistical software JMP 13 (SAS Institute). In this DoE approach, the three-factor Box–Behnken design was used to be suitable for second-order models, which was composed of 15 preparation runs. The design is cited as a common experimental design for screening crucial factors. In this design, all factors (lipid concentration, FRR, and TFR) have three levels: low, center, and high. In addition, three center samples were included in this design and used as a source for error estimation. It was important to assign the appropriate level ranges to each of the factors as they should be neither too close nor too far away from each other. This reduces the probability to miss the optimal effect. The levels of each factor were set based on past reports. Multiple regression analysis applying a least-squares error estimation. It was important to assign the appropriate level ranges to each of the factors as they should be neither too close nor too far away from each other. This reduces the probability to miss the optimal effect. The levels of each factor were set based on past reports. Multiple regression analysis applying a least-squares error estimation. It was important to assign the appropriate level ranges to each of the factors as they should be neither too close nor too far away from each other. This reduces the probability to miss the optimal effect. The levels of each factor were set based on past reports.

### Statistical Analysis.
All values were indicated as mean ± standard deviation. Statistical comparisons between two conditions were performed using the Student’s t-test.

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**Figure 1.** Effect of pH during dialysis on the particle size (filled circle) and PDI (empty circle) of empty LNPs (A) and LNP-siRNA systems (B). Empty LNPs and LNP-siRNA suspensions injected into microdialysis tubing, Spectrum Labs, Rancho Dominguez, CA.
performed using paired Student’s t-test. Values of $P < 0.05$ were considered to indicate statistical significance.

### RESULTS AND DISCUSSION

**Effect of pH on the Particle Size and PDI of Empty LNPs and LNP-siRNA Systems.** LNP-siRNA formed with the use of a microfluidic device are typically dialyzed in a neutral buffer to remove ethanol and raise the pH to physiological values. A recent study has revealed that in both empty LNP and LNP-siRNA systems, the ionizable lipid Dlin-KC2-DMA is neutralized during dialysis in PBS pH 7.4, leading to the formation of a destabilized lipid bilayer structure that promotes particle fusion.6 In our DoE approach, we first verified that a similar fusion can be induced in empty LNPs and LNP-siRNA systems containing the ionizable lipid DODAP by measuring the particle size over the range of pH 4.0 to pH 7.4 compared to PBS (Figure 1). Both empty LNPs and LNP-siRNA systems showed an increase in particle size starting at around pH 6.5, suggesting that particle fusion had occurred. This is expected since the apparent pKₐ of DODAP has been reported to be 6.58,18,19 and as the pH is raised above physiological values. A recent study has revealed that in both empty LNPs and LNP-siRNA systems containing the ionizable lipid DODAP, the increase in particle size following dialysis was observed with both empty LNPs and LNP-siRNA systems. As the FRR was increased (i.e., more aqueous), a reduction in the particle size and PDI,8 which is in agreement with the results of this study. This highlights the importance of the ethanol-to-aqueous ratio when controlling the physical properties of LNP systems produced by microfluidics. Furthermore, in the case of LNP-siRNA systems, the lipid concentration showed a significant effect on the particle size and PDI. Since the charge ratio (N/P) in the prepared LNP-siRNA systems was fixed at 3, the concentration of siRNA was also modified as to maintain a constant lipid-to-siRNA ratio. Therefore, it is expected that as the physical characteristics of LNP-siRNA systems are affected by the local concentration of DODAP/siRNA binding at the region where the two fluids mix in a microfluidics device, higher concentrations of particles (in the presence of the solvent) lead to increased and unintended particle fusion.

**Multiple Regression Modeling Based on DoE Approach.** We next investigated the effects of preparation parameters (lipid concentration, FRR and TFR), dialysis process, and complex formation between siRNA and ionizable cationic lipids on the resulting properties of empty LNP and LNP-siRNA systems using a multiple regression analysis. Instead of a full-factorial design, the Box–Behnken approach was used as it was suited to create a quadratic model. A three-factor Box–Behnken design is almost rotatable, which means that all design points are at the same distance from the center of the design. Such a design lends itself to aptly create a response surface plot as the prediction error is the same for all design points.27 The statistical approach using DoE to design the optimal properties of LNPs has been investigated in many studies, and the usefulness of this approach has been demonstrated in the development of LNP-related product formulations.4,17,28

In this DoE study, the preparation parameters are shown in Table 1. Since siRNA encapsulation values showed approximately 90% in all LNP-siRNA systems (Supporting Information, Figure S1), the particle size and PDI were selected as physicochemical properties to be evaluated further in this study. First, the accuracy of the multiple regression equations constructed by this design was evaluated. As shown in the scatterplots of experimental versus predicted values (Supporting Information, Figure S2), the coefficients of determination adjusted by the degrees of freedom ($R^2$) were high. The $R^2$ values for particle size and PDI of empty LNPs before dialysis were 0.94 and 0.99, respectively, whereas these values after dialysis were 0.99 and 0.92, respectively. Similarly, the $R^2$ values for particle size and PDI of siRNA-containing systems before dialysis were 0.96 and 0.95, respectively, and 0.91 and 0.95, respectively, after dialysis. Additionally, the mean of the experimental data (blue line) fell outside the bounds of the 95% confidence area (pink area), indicating that the overall regression model was statistically significant.

**Evaluation of Statistically Significant Preparation Parameters.** We then evaluated this data to further understand the impact of lipid concentration, FRR, and TFR on the particle size and PDI of these systems. As detailed in the methods, the three parameters tested provide for nine variables that affect the outcomes (particle size and PDI). It can be seen from Table 2 that FRR and FRR × FRR showed a remarkable effect ($P < 0.01$) on the particle size and PDI of both empty LNPs and LNP-siRNA systems. Previous work using LNPs composed of 1,2-dioleoyl-3-trimethylammonium-propane and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine also reported that FRR in the range of 1 to 5 significantly affected the particle size and PDI,8 which is in agreement with the results of this study. This highlights the importance of the ethanol-to-aqueous ratio when controlling the physical properties of LNP systems produced by microfluidics. Furthermore, in the case of LNP-siRNA systems, the lipid concentration showed a significant effect on the particle size and PDI. Since the charge ratio (N/P) in the prepared LNP-siRNA systems was fixed at 3, the concentration of siRNA was also modified as to maintain a constant lipid-to-siRNA ratio. Therefore, it is expected that as the physical characteristics of LNP-siRNA systems are affected by the local concentration of DODAP/siRNA binding at the region where the two fluids mix in a microfluidics device, higher concentrations of particles (in the presence of the solvent) lead to increased and unintended particle fusion.

**Model Analysis against the Properties of Empty LNPs and LNP-siRNA Systems.** The impact on the statistically significant effect of preparation parameters (lipid concentration, FRR, and TFR) on the particle size and PDI of empty LNPs and LNP-siRNA systems before and after dialysis was further investigated in a resonance surface model using our DoE analysis. The response surface factors against the particle size of empty LNPs and LNP-siRNA systems before and after dialysis are shown in Figures 2 and 3, respectively. An overall trend of increase in particle size following dialysis was observed with both empty LNPs and LNP-siRNA systems. As the FRR was increased (i.e., more aqueous), a reduction in the particle size on both empty LNPs and LNP-siRNA systems was detected. This is likely because FRR strongly affects the polarity throughout the chamber as well as the final ethanol concentration, resulting in the change of exchange rates for individual lipid molecules. In the studies using hydrodynamic flow-focusing techniques, the increase in the FRR has been

### Table 1. DoEs for DoE-Based Microfluidic Flow Settings

<table>
<thead>
<tr>
<th>run no.</th>
<th>lipid conc. (mM)</th>
<th>FRR (vol/vol)</th>
<th>TFR (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
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</tr>
<tr>
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<td>20</td>
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</tr>
<tr>
<td>5</td>
<td>10</td>
<td>1</td>
<td>2</td>
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<td>6</td>
<td>30</td>
<td>1</td>
<td>2</td>
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<td>7</td>
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<td>2</td>
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<td>9</td>
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<td>20</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>20</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

*Different lipid concentrations, FRR, and TFR used for the preparation of each empty LNP and LNP-siRNA system in this study are represented.*
reported to decrease the liposome particle size,\textsuperscript{29,30} similar to what was observed in this study. On the other hand, our mathematical model showed that lipid concentration in fluid-enced (with statistical significance) the particle size of LNP-siRNA systems but not empty LNPs (Table 2). In the model analysis, it was observed that the particle size of LNP-siRNA systems was affected by the FRR and lipid concentration and confirmed that the particle size will likely increase if a low FRR and a high lipid concentration are used.

Figures 2 and 3 show the response surface factors against the PDI and particle size of empty LNPs and LNP-siRNA systems before and after dialysis. The effect of lipid concentration (mM) and FRR (vol/vol) on the particle size before dialysis at TFR (mL/min) = 2 (A), the effect of lipid concentration and FRR on the particle size after dialysis at TFR = 2 (B), the effect of FRR and TFR on the particle size before dialysis at lipid concentration = 20 (C), the effect of FRR and TFR on the particle size after dialysis at lipid concentration = 20 (D), the effect of lipid concentration and TFR on the particle size before dialysis at FRR = 3 (E), and the effect of lipid concentration and TFR on the particle size after dialysis at FRR = 3 (F).

Table 2. Effect of Each Factor against PDI and Particle Size of Empty LNPs and LNP-siRNA Systems

<table>
<thead>
<tr>
<th>factors</th>
<th>empty LNPs</th>
<th></th>
<th>LNP-siRNA systems</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>particle size</td>
<td>PDI</td>
<td>particle size</td>
<td>PDI</td>
</tr>
<tr>
<td></td>
<td>before dialysis</td>
<td>after dialysis</td>
<td>before dialysis</td>
<td>after dialysis</td>
</tr>
<tr>
<td>lipid conc.</td>
<td>0.044</td>
<td>0.879</td>
<td>0.187</td>
<td>0.271</td>
</tr>
<tr>
<td>FRR</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.009</td>
</tr>
<tr>
<td>TFR</td>
<td>0.138</td>
<td>0.923</td>
<td>0.154</td>
<td>0.181</td>
</tr>
<tr>
<td>lipid conc. × lipid conc.</td>
<td>0.839</td>
<td>0.482</td>
<td>0.005</td>
<td>0.704</td>
</tr>
<tr>
<td>FRR × FRR</td>
<td>&lt;0.001</td>
<td>0.006</td>
<td>&lt;0.001</td>
<td>0.004</td>
</tr>
<tr>
<td>TFR × TFR</td>
<td>0.452</td>
<td>0.599</td>
<td>0.036</td>
<td>0.077</td>
</tr>
<tr>
<td>lipid conc. × FRR</td>
<td>0.083</td>
<td>0.422</td>
<td>&lt;0.001</td>
<td>0.032</td>
</tr>
<tr>
<td>lipid conc. × TFR</td>
<td>0.874</td>
<td>0.793</td>
<td>0.459</td>
<td>0.969</td>
</tr>
<tr>
<td>FRR × TFR</td>
<td>0.563</td>
<td>0.074</td>
<td>0.022</td>
<td>0.371</td>
</tr>
</tbody>
</table>
3) was associated with a decreased PDI in both empty LNPs and LNP-siRNA systems. However, in the range of 3–5 FRR, no impact on PDI was observed. In addition, lipid concentration was found to have a greater impact on the PDI of LNP-siRNA systems than on that of empty LNPs, and there was a tendency for the PDI to increase with increasing lipid concentration. Interestingly, a reduction in the PDI was observed following dialysis in LNP-siRNA systems.

**Comparative Evaluation of DoE Results for Empty LNPs and LNP-siRNA Systems before and after Dialysis.**

The experimental results of this DoE approach (outlined in Table 1) are shown in Figure 6 to compare changes in the particle size and PDI before and after dialysis between empty LNPs and LNP-siRNA systems. Two different trends were identified between empty LNPs and LNP-siRNA systems. First, LNP-siRNA systems showed substantial reductions in the PDI following dialysis, while the PDI of empty LNPs generally remained the same or slightly increased following dialysis. Furthermore, the increase in the particle size that occurred after dialysis was much higher with LNP-siRNA systems than with empty LNPs. This observation is consistent with previous data that showed loaded systems (at FRR = 3) display generally larger sizes than the unloaded formulation and that the presence of siRNA should contribute to increasing particle size.

The second trend related to the change in particle size and PDI as a result of FRR. Empty LNPs displayed a similar small particle size at FRR = 3 and 5; however, the particle sizes (and PDI) before and after dialysis were much larger at FRR = 1. In contrast, for LNP-siRNA, the particle sizes stayed relatively consistent before dialysis regardless of FRR. A similar observation was made for the post-dialysis samples, where all formulations displayed similar sizes that are generally larger than their predialysis counterparts. This indicates that the presence of anionic cargo such as siRNA limits the rearrangement of lipids. Consistent with previous observations, we believe that the dramatic differences for empty LNPs were a result of substantial lipid reorganization.

**Effect of pH Neutralization in the Presence or Absence of Ethanol.** LNP-siRNA suspensions prepared at FRR = 1 (with 50% ethanol v/v) showed little change in the particle size and PDI before and after dialysis. In order to study the effect of pH neutralization in the presence of ethanol on the particle size and PDI of LNP-siRNA, two formulation processes were tested. LNP-siRNA produced at FRR = 3 and 1 were collected and neutralized by (1) dialysis against PBS or (2) by injection into microfluidics with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.0) containing 200 mM sodium chloride followed by dialysis. The composition of the HEPES buffer was adjusted to show a salt concentration and pH similar to those of PBS after
the injection into the microfluidics. As shown in Figure 7A, LNP-siRNA prepared at FRR = 3 showed an increase in particle size and a decrease in PDI after neutralization, but there was no difference between the two pH neutralization methods. On the other hand, as shown in Figure 7B, in LNP-siRNA suspensions with FRR = 1, a significant increase in the particle size and a decrease in the PDI were observed after pH neutralization using the microfluidic mixer. This relationship between the FRR and particle morphology is consistent with similar observations for particles composed of DSPC/Chol/PEG-DSPE (52/45/3 mol %) prepared using the same technique at FRRs of 1 and 3 (Supporting Information, Figure S3). It suggests that higher FRRs produce larger particle sizes even when the protonation states of ionizable lipids are not convoluting factors to the size and morphology. High ethanol contents provide an environment with relatively high viscosity, osmolality, and lipid solubility, which are supportive of uncontrolled particle fusion. In addition, it is likely that the LNP metamorphosis that occurs as the pH is neutralized is a shear-sensitive process leading to a particle size increase. Although further studies are needed, our findings suggest that the method of pH neutralization process in manufacturing is important to obtain LNP-siRNA with desirable properties (defined particle sizes, low polydispersity indices, and high entrapment).

**Effect of the Binding of siRNA with DODAP on the Particle Size of siRNA-LNP Systems.** The increase in the particle size with decreasing FRR (i.e., more ethanol) was suppressed in the presence of siRNA as compared to the case of empty LNPs. Therefore, it is considered that small vesicles

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**Figure 6.** Comparative evaluation of DoE results for empty LNPs and LNP-siRNA systems before (white bar) and after dialysis (gray bar): PDI before and after dialysis of empty LNPs (A) and LNP-siRNA systems (B) and particle size before and after dialysis of empty LNPs (C) and LNP-siRNA systems (D). For formulation parameters of each run, please refer to Table 1. Results indicate mean ± standard deviation (n = 3). **P < 0.05, ***P < 0.01, and ****P < 0.001.

**Figure 7.** Effect of ethanol content on the change of particle size (solid bar) and PDI (closed circle) in different pH neutralization methods. LNP-siRNA suspensions were prepared under the condition of FRR = 3 (A) and 1 (B) by microfluidics. Each suspension was divided to neutralize by (1) dialysis with PBS or (2) by injection into microfluidics with 50 mM HEPES buffer solution (pH 8.0). The changes in particle size and PDI before and after neutralization of the suspension prepared at FRR = 3 and 1 were compared for different neutralization methods. Results indicate mean ± standard deviation (n = 3).
which contain siRNA between closely apposed lipid monolayers are not affected by the ethanol concentration because of their low solubility in ethanol. In addition to this, we have previously shown that “free” ionizable lipid (i.e., not interacting with nucleic acid) contributes to particle rearrangement and that ionizable lipid interacting with nucleic acid does not support rearrangement. With high ethanol ratios (low FRR), empty LNPs showed a remarkable increase of particle size, likely due to increased solubility of all lipid components in the high ethanol content solution.

In order to show that the formation of small vesicles with anionic polymers is related to the change in particle size with low FRR, complexes with HA with molecular weight similar to that of siRNA were prepared, and the particle size was evaluated. HA has previously been reported to form complexes with cationic liposomes in a manner similar to nucleic acids, although the acidity of its carboxylic acid is relatively lower than that of phosphoric acid in siRNA. The experiments were performed using the same DoE approach as in the comparative studies of empty LNP with LNP-siRNA systems, and the results were analyzed as resonance surface modeling based on z-average values. As expected, LNP-HA systems (LNP containing HA) demonstrated that the degree of change in particle size caused by FRR was smaller than that of empty LNPs but larger than that of LNP-siRNA systems, suggesting that the binding affinity of small vesicles with anionic cargos plays a significant role in the formulation of the LNP complexes (Figure 8). Further studies are required to determine how different types of anionic cargos (modified nucleic acids or peptides) influence nanoparticle formation.

## CONCLUSIONS

In this paper, since little has been reported on the evaluation of the effect of manufacturing parameters on the particle size by tracing from microfluidics to dialysis, the effect of microfluidics parameters and subsequent dialysis on the physicochemical properties of empty LNPs and LNP-siRNA systems was elucidated by statistical evaluation using a DoE approach. The preparation parameters (lipid concentration, FRR, and TFR) were evaluated, and lipid concentration and FRR were identified as the critical process parameters to be monitored in order to achieve the desired and robust LNP-siRNA particles. We believe that such a study contributes critical and useful information to the generation of a design space for manufacturing parameters in the process development of LNP-siRNA systems. Furthermore, we demonstrated that, in each manufacturing step from rapid mixing to dialysis, the presence of siRNA dramatically impacts the physicochemical properties of LNP-siRNA systems. The results and discussions above are expected to enable robust commercial production from lab-on-a-chip scale not only for LNP-siRNA systems but also for new formulations of LNP systems with other anionic cargos such as mRNA and DNA.

## ASSOCIATED CONTENT

*Supporting Information*

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.langmuir.0c03039.

Encapsulation efficiency of siRNA/LNP systems prepared by microfluidics in the DoE approach; scatterplots of experimental vs predicted values modeled by multiple regression based on DoE for PDI and particle size; and effect of FRR on the morphology of particles composed of DSPC/Chol/PEG-DSPE (52/45/3 mol %) (PDF)

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**Figure 8.** Response surface factors against particle size of empty LNPs (A), LNP-HA complexes (B), and LNP-siRNA systems (C) after dialysis: the effect of FRR (vol/vol) and TFR (mL/min) on the particle size at lipid concentration = 20 mM. In the preparation of LNP-HA complexes, HA was dissolved at a charge ratio of DODAP nitrogen/HA carboxylic acid (N/COOH) = 3 in acetate buffer.
Author Contributions
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes
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ABBREVIATIONS
LNPs, lipid nanoparticles; FRF, flow rate ratio; TFR, total flow rate; DoE, design of experiments; PDI, polydispersity index; HSPC, hydrogenated soy phosphatidylcholine; PEG-DSPPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000]; Chol, cholesterol; DODAP, 1,2-dioleoyl-3-dimethylammonium propane; SHM, staggered herringbone micromixer; PBS, phosphate buffered saline; HA, hyaluronic acid; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane.

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