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## Production of siRNA-Loaded Lipid Nanoparticles Using a Microfluidic Device

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**TITLE:**

Production of siRNA-Loaded Lipid Nanoparticles Using a Microfluidic Device

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**SUMMARY:**

Microfluidic-based lipid nanoparticle (LNP) production methods have attracted attention in drug delivery systems (DDSs), including RNA delivery. This protocol describes the fabrication, LNP (siRNA-loaded LNP) production, and LNP evaluation processes using our original microfluidic device named iLiNP.

**ABSTRACT:**

The development of functional lipid nanoparticles (LNPs) is one of the major challenges in the field of drug delivery systems (DDS). Recently, LNP-based RNA delivery systems, namely, RNA-loaded LNPs have attracted attention for RNA therapy. In particular, mRNA-loaded LNP vaccines were approved to prevent COVID-19, thereby leading to the paradigm shift toward the development of next-generation nanomedicines. For the LNP-based nanomedicines, the LNP size is a significant factor in controlling the LNP biodistribution and LNP performance. Therefore, a precise LNP size control technique is indispensable for the LNP production process. Here, we report a protocol for size controlled LNP production using a microfluidic device, named iLiNP. siRNA loaded LNPs are also produced using the iLiNP device and evaluated by *in vitro* experiment. Representative results are shown for the LNP size, including siRNA-loaded LNPs, Z-potential, siRNA encapsulation efficiency, cytotoxicity, and target gene silencing activity.

## INTRODUCTION:

Lipid nanoparticle (LNP) is one of the most widely used nanocarriers for RNA delivery systems. Recently, mRNA-loaded LNPs have been approved as vaccines for the prevention of COVID-19<sup>1-3</sup>. Generally, the size of LNP plays a crucial role in the biodistribution and drug delivery systems (DDS) performance, including gene silencing or protein expression<sup>4-6</sup>. Therefore, a precise LNP size control method is required for the LNP production process.

For the production of size controlled LNPs, microfluidic devices have attracted attention over the years<sup>7</sup>. In 2018, the first Food and Drug Administration (FDA)-approved siRNA-loaded LNPs (e.g., Onpattro) was developed using the microfluidic device<sup>8,9</sup>. In the microfluidic-based LNP production method, a lipid solution and an aqueous solution are introduced separately into the microfluidic device, and then mixed in the microchannel. To enhance the mixing efficiency, the chaotic mixer device has been used for the LNP production<sup>10-12</sup>. The chaotic mixer device makes it possible to produce specific-sized LNPs.

A simple microfluidic device, named invasive lipid nanoparticle production (iLiNP), equipped with baffle structures, has been developed to control the LNP size precisely<sup>13,14</sup>. In comparison with the chaotic mixer device, the iLiNP device was able to control the LNP size ranged from 20 to 100 nm at 10 nm intervals. In addition, the iLiNP device produced siRNA-loaded LNPs<sup>6</sup>, mRNA-loaded LNPs<sup>15</sup>, ribonucleoprotein-loaded LNPs<sup>16</sup>, and exosome-like LNPs<sup>17</sup>. The aim of this paper is to introduce the fabrication and siRNA-loaded LNP production process of the iLiNP device and describe the LNP evaluation process produced by the iLiNP device.

## PROTOCOL:

### 1. Fabrication of the iLiNP device

NOTE: The iLiNP device is fabricated using the standard soft lithography method<sup>18</sup>. The detailed fabrication protocol was reported previously<sup>10,13</sup>.

#### 1.1. SU-8 mold fabrication

1.1.1. Pour SU-8 3050 onto a 3-inch silicon wafer. Spin coat the silicon wafer to obtain a 100  $\mu$ m thick SU-8 layer.

1.1.2. Bake the silicon wafer by placing on a hotplate at 65 °C for 5 min and 95 °C for 45 min.

1.1.3. After baking, place the silicon wafer on the stage of a desktop maskless lithography system.

1.1.4. Expose the silicon wafer to UV light at 365 nm for 1.5 s per one position.

NOTE: A desktop maskless lithography system was used in this experiment. The system automatically exposes UV light at a divided irradiation area (one position) of the microchannel.

1.1.5. After UV irradiation, bake the silicon wafer on the hotplate at 65 °C for 1 min and 95 °C for 5 min.

1.1.6. Cool the silicon wafer, and then soak in a SU-8 developer for 15 min to remove unexposed SU-8.

1.1.7. Treat the SU-8 mold with vapor of Trichloro(1H,1H,2H,2H-perfluorooctyl)silane using a desiccator and vacuum pump.

## 1.2. Fabrication of the iLiNP device

1.2.1. Mix the silicone base and polydimethylsiloxane (PDMS) curing agent in a 10:1 ratio (w/w).

1.2.2. Degas the mixture using a vacuum pump and desiccator.

NOTE: PDMS was degassed using a vacuum pump for 10 min at room temperature.

1.2.3. Pour the degassed PDMS onto the SU-8 mold in a 100 mm Petri dish up to 0.5 to 1 cm thickness, followed by baking in an oven at 80 °C for 1 h.

1.2.4. Cool the mold, and then peel the PDMS substrate from the SU-8 mold using a tweezer.

1.2.5. Punch three holes (0.5 mm) in the PDMS substrate. Bond the PDMS substrate and a glass slide using an oxygen plasma cleaner to build an iLiNP device (see **Figure 1**)<sup>13</sup>.

1.2.6. Connect three PEEK capillaries (I.D. 0.3 mm, O.D. 0.5 mm) to the inlets and outlet of the iLiNP device and cure with a superglue.

NOTE: The length of the PEEK capillaries is adjustable and depends on the experiment.

## 2. Preparation of lipid solutions

2.1. Prepare lipid/ethanol solutions: 13.4 mM 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 10 mM 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 20 mM 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP), 5 mM 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2k), and 20 mM cholesterol. Store the stock solutions at -20 °C prior to the experiment.

2.2. To produce the siRNA-loaded LNPs, mix DOTAP, DSPC, cholesterol, and DMG-PEG2k solutions at a molar ratio of 50/10/38.5/1.5. The total lipid concentration is adjusted to 8 mM.

## 3. Preparation of aqueous solutions

133  
134 3.1. Prepare aqueous solutions: 154 mM NaCl (saline), 25 mM acetate buffer at pH 4.0 using  
135 DNase/RNase-free distilled water.

136  
137 3.2. Filter the solutions through 0.2  $\mu$ m sized membrane filters or syringe filters.

#### 138 139 **4. Preparation of the siRNA/buffer solution**

140  
141 4.1. Dissolve 70  $\mu$ g of siGL4 into 1 mL of 25 mM acetate buffer (pH 4.0).

142  
143 NOTE: siGL4 is used for the knockdown of luciferase gene.

#### 144 145 **5. Set up of the iLiNP device and production of LNPs**

146  
147 NOTE: See **Figure 1** for the schematics.

148  
149 5.1. Fill 1 mL glass syringes with lipid and aqueous solutions (from steps 3.1 and 4.1 in  
150 individual syringes), respectively.

151  
152 NOTE: Adjust the lipid and aqueous solution volume depending on the amount required for the  
153 LNP evaluation experiment.

154  
155 5.2. Connect the glass syringes to the PEEK capillaries using syringe connectors.

156  
157 5.3. Set the flow rate of the lipid and aqueous solutions.

158  
159 NOTE: The flow rate ratio (FRR) of the aqueous phase to the lipid phase is ranged from 3:1 to  
160 9:1.

161  
162 5.4. Introduce the lipid and aqueous solutions separately into the iLiNP device using syringe  
163 pumps.

164  
165 5.5. Collect LNP suspensions in a microtube from the outlet of the iLiNP device (**Figure 1**).

#### 166 167 **6. Dialysis of the LNP suspension and LNP size measurement**

168  
169 6.1. Dialyze the LNP suspension using a dialysis membrane (12–14 kDa MW cutoffs) at 4 °C  
170 overnight against saline or D-PBS for POPC LNPs and siRNA-loaded LNPs, respectively.

171  
172 NOTE: POPC is used as a model lipid in 154 mM of aqueous saline.

173  
174 6.2. Collect the dialyzed LNP suspensions in microtubes.

175  
176 6.3. Pipette 20–30  $\mu$ L of the LNP suspension to a micro quartz cell.

6.4. Measure the LNP size, LNP size distribution, and polydispersity index by dynamic light scattering (DLS).

## 7. Measurement of Z-potential of the LNP

NOTE: For the measurement of Z-potential, a particle analyzer (see **Table of Materials**) was used following the manufacturer's instruction.

7.1. Dilute the LNP suspension obtained from step 6.1, 35 times with 10 mM HEPES buffer (pH 7.4).

7.2. Pipette 700 to 1000  $\mu$ L of the diluted LNP suspension to a capillary cell.

7.3. Measure the Z-potential as per the manufacturer's instruction.

## 8. siRNA encapsulation efficiency by RiboGreen assay

NOTE: Ribogreen assay is performed to evaluate the siRNA encapsulation into LNPs<sup>19</sup>.

Ribogreen assay can measure the amount of RNAs inside and outside of LNPs with/without a surfactant (e.g., TritonX-100).

8.1. Dilute 2 mg/mL of siGL4 with 10 mM HEPES buffer (pH 7.4) to 500 ng/mL siGL4 solution.

8.2. Prepare the dilution series (0, 12.5, 25, 50, 100, 200 ng/mL) of siGL4 solution to make a calibration curve for Triton (+) and Triton (-) samples.

8.3. Dilute the LNP suspension 100 times with 10 mM HEPES buffer (pH 7.4).

8.4. Mix the following for Triton (+) solution: 980  $\mu$ L of 10 mM HEPES (pH 7.4), 20  $\mu$ L of 10% w/v TritonX-100, and 1.25  $\mu$ L of RiboGreen for 10 wells of a 96-well microplate.

8.5. Mix the following for Triton (-) solution: 1000  $\mu$ L of 10 mM HEPES (pH 7.4) and 1.25  $\mu$ L of RiboGreen for 10 wells of a 96-well microplate.

8.6. Pipette 100  $\mu$ L of the dilution series of siGL4 solution and diluted LNP suspensions into the wells of a black 96-well microplate.

NOTE: The dilution series of siGL4 solution and diluted LNP suspensions were dispensed into four microwells per condition.

8.7. Pipette 100  $\mu$ L of the detection solution (TritonX-100 (+) or Triton (-)) into the wells.

NOTE: The detection solution (TritonX-100 (+)) was dispensed into two wells per sample per

condition, and the TritonX-100 (-) solution was dispensed into the remaining two wells per sample condition.

8.8. Incubate the microplate for 5 min at room temperature.

8.9. Measure the fluorescence intensity using a microplate reader at a wavelength of 475 nm.

8.10. Calculate the siRNA encapsulation efficiency from the following equation<sup>19</sup>.

$$\text{siRNA encapsulation efficiency [\%]} = \frac{\text{siRNA concentration of Triton (+)} - \text{siRNA concentration of Triton (-)}}{\text{siRNA concentration of Triton (+)}} \times 100$$

## 9. Cell culture

9.1. Prepare a growth medium containing DMEM, heat-inactivated 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 400 µg/mL G418.

9.2. Culture HeLa cells stably expressing firefly and Renilla luciferase (HeLa-dluc) in a 100 mm TC-treated cell culture dish containing the growth medium at 37 °C in a 5% CO<sub>2</sub> incubator.

## 10. Cell viability assay

10.1. Seed 100 µL of a suspension of HeLa cells in the growth medium (6 x 10<sup>3</sup> cells/well) in a 96-well microplate.

NOTE: Cells were counted using a cell counter plate and a microscope.

10.2. Incubate the microplate for 24 h at 37 °C in a 5% CO<sub>2</sub> incubator.

10.3. Dilute the siRNA-loaded LNPs with DMEM (FBS (-)) at the concentrations of 1, 20, and 50 nM siRNAs.

10.4. Dispense 100 µL of the diluted siRNA-loaded LNP suspension per well.

10.5. Incubate the microplate for 4 h at 37 °C in a 5% CO<sub>2</sub> incubator.

10.6. Remove the LNP suspension and add 100 µL of DMEM (FBS (+)).

10.7. Incubate the microplate for 20 h at 37 °C in a 5% CO<sub>2</sub> incubator.

10.8. Measure the cell viability using a commercially available kit according to the

manufacturer's protocol.

NOTE: D-PBS (-) was used as the negative control.

## **11. Luciferase gene knockdown assay**

11.1. Seed 75  $\mu$ L of a suspension of HeLa cells in the growth medium ( $4.5 \times 10^3$  cells/well) in a 96-well microplate.

11.2. Incubate the microplate for 24 h at 37 °C in a 5% CO<sub>2</sub> incubator.

11.3. Dilute the siRNA-loaded LNPs with DMEM (FBS (-)) at the concentrations of 1, 20, and 50 nM siRNAs.

11.4. Dispense 75  $\mu$ L of the diluted siRNA-loaded LNP suspension per one well.

11.5. Incubate the microplate for 4 h at 37 °C in a 5% CO<sub>2</sub> incubator.

11.6. Remove the LNP suspension and add 75  $\mu$ L of DMEM (FBS (+)).

11.7. Incubate the microplate for 20 h at 37 °C in a 5% CO<sub>2</sub> incubator.

11.8. Measure the luciferase expression using a commercially available kit according to the manufacturer's protocol.

NOTE: We used D-PBS (-) as the negative control.

## **REPRESENTATIVE RESULTS:**

**Figure 2A,B** shows the POPC LNP size distribution produced at different flow conditions. The microfluidic-based LNP preparation method can control the size of LNPs by the flow conditions such as the total flow rate (TFR) and the FRR. Compared with the typical microfluidic devices, including the chaotic mixer device and the flow-focusing microfluidic device, the iLiNP device enabled precise LNP size control ranging from 20 to 100 nm (**Figure 2**). Small-sized LNPs are formed at high total flow rate conditions. In addition, the LNP sizes formed at the FRR of 5 were smaller than those of the FRR of 3, regardless of the total flow rate<sup>13</sup>.

siRNA-loaded LNPs were also prepared using the iLiNP device (**Figure 3A**). For the siRNA-loaded LNP preparation, DOTAP, a cationic lipid, was used to encapsulate the siRNA into the LNPs effectively. The iLiNP device produced 90 nm sized siRNA-loaded cationic LNPs with narrow distribution (**Figure 3A,B**). The siRNA encapsulation efficiency was 95% because of the electrostatic interaction between the cationic lipid and negatively charged siRNAs (**Figure 3C**).

Cytotoxicity and the gene silencing activity of 90 nm sized siRNA-loaded LNPs were evaluated as shown in **Figure 4** and **Figure 5**. siRNA-loaded LNPs did show cytotoxicity at a dose of 10 and 100



nM siRNA. We also confirmed that the expression level of luciferase was decreased depending on the siRNA concentration. The siRNA-loaded LNPs suppressed 80% luciferase expression at a dose of 100 nM siRNA. The effect of LNP size on the gene silencing activity was reported previously<sup>6,13,17</sup>.

#### FIGURE AND TABLE LEGENDS:

**Figure 1: (A) Schematic illustration and (B) photograph of the iLiNP device.** The iLiNP device comprises PDMS and glass substrates. The iLiNP device is connected to PEEK capillaries with a superglue. The lipid and siRNA/buffer solutions are separately introduced into the iLiNP device using syringe pumps. The LNP suspension is collected in a microtube.

**Figure 2: POPC LNP size distributions produced by the iLiNP device at the different flow rate ratios (FRR).** The POPC LNP size is measured by dynamic light scattering (DLS). The POPC LNPs are prepared by changing the total flow rate and the FRR: (A) 3 FRR and (B) 5 FRR. Small-sized LNPs are formed at high total flow rate conditions. In addition, the LNP sizes formed at the FRR of 5 were smaller than those at the FRR of 3.

**Figure 3: Characterization of the siRNA-loaded LNPs.** (A) Size distribution of siRNA-loaded LNPs. siRNAs (siGL4) are encapsulated into the LNPs by electrostatic interaction between the cationic lipid (DOTAP) and negatively-charged siRNAs. (B) Z-potential of the siRNA-loaded LNPs. The LNP suspension was diluted with 10 mM HEPES buffer (pH 7.4) before the measurement. Data are represented as mean  $\pm$  SD (Standard Deviation).  $n = 3$ . (C) siRNA encapsulation efficiency of the DOTAP-based LNPs. The encapsulation efficiency was determined by RiboGreen assay. Data are represented as mean  $\pm$  SD.  $n = 3$ .

**Figure 4: Cytotoxicity of the siRNA-loaded LNPs.** siRNA-loaded LNPs were diluted with DMEM (FBS (-)) to obtain the siGL4 concentrations of 10 and 100 nM. The LNP suspensions are added to HeLa-dLuc cells and incubated for 4 h at 37 °C in a 5% CO<sub>2</sub> incubator. N.T.: Non-treated (D-PBS(-)). Data are represented as the mean  $\pm$  SD.  $n = 3$ .

**Figure 5: Luciferase gene knockdown activity treated with siRNA-loaded LNPs.** siRNA-loaded LNPs are prepared in the same manner as cell viability assay. The luciferase expression level is measured using Dual-Glo Luciferase Assay System. N.T.: Non-treated (D-PBS(-)). Data are represented as mean  $\pm$  SD.  $n = 3$ .

#### DISCUSSION:

The LNP size affects the LNP biodistribution, anti-tumor effect, and gene silencing performance. Therefore, the LNP size control method is a significant technique for producing DDS nanomedicines, including RNA delivery systems. The aim of this paper is to introduce the iLiNP device for precise size tuning of LNPs and its application to the siRNA-loaded LNPs production. The iLiNP device was able to control the LNP size ranged from 20 to 100 nm (**Figure 2**)<sup>13</sup>. When the flow conditions, such as the total flow rate and the FRR are changed to control the LNP size, the LNP suspension should be collected after about 5 to 10 s to stabilize the solution flow. The LNP suspension collected from the outlet of the iLiNP device was dialyzed immediately against

the buffer solution to remove ethanol and prevent LNP aggregation.

The LNP size control is one of the major challenges in the field of DDS. Generally, the conventional LNP production process, such as the lipid film hydration method, needs a size tuning process after the LNP production<sup>20</sup>. On the other hand, the microfluidic-based LNPs production method can produce the size-controlled LNPs by introducing the lipid and aqueous solutions into the microfluidic device<sup>6,11,13</sup>. Although the dialysis process is required to remove ethanol from the LNP suspension, a continuous process by the microfluidic device coupled with the tangential flow system promises the automation of the LNP production process<sup>14</sup>. According to the literature, the POPC LNP sizes were 50–60 nm and 30–60 nm, for the flow-focusing microfluidic device<sup>21</sup> and the chaotic mixer device, respectively<sup>10</sup>. Compared with other microfluidic devices, the iLiNP device enables the POPC LNP size control in a wide range from 20 to 100 nm.

The fabrication process of the iLiNP device employed was the standard soft lithography. Thus, the iLiNP device can be mass-produced by rapid prototyping technique and prevent cross-contamination of solutions by using a disposable device. The iLiNP device can produce siRNA-loaded LNPs in the same manner as the POPC LNP production method. For the LNP production method using the iLiNP device, the user does not require any complicated procedures. For these reasons, the microfluidic-based LNP production method, including the iLiNP device, will be expected to be employed as the standard LNP production method. The protocol of this paper can be adapted to other microfluidic devices for LNP production. In addition, the production of mRNA-loaded LNPs is also enabled by changing the siRNA/buffer solution to a buffer solution containing mRNAs.

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#### DISCLOSURES:

The authors have nothing to disclose.

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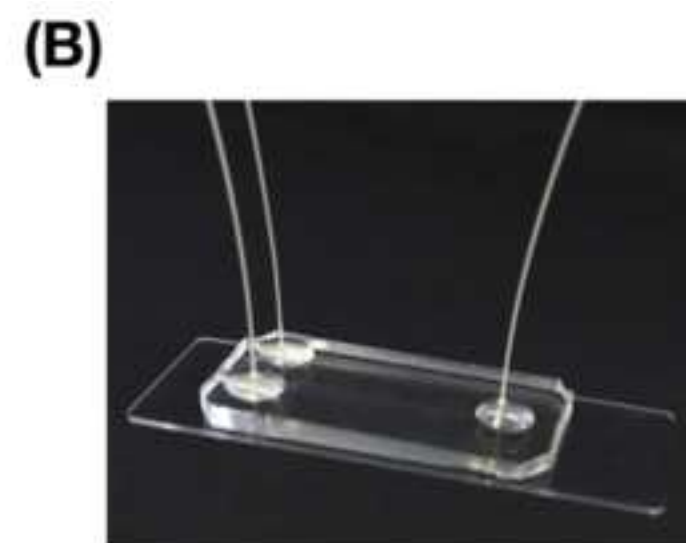
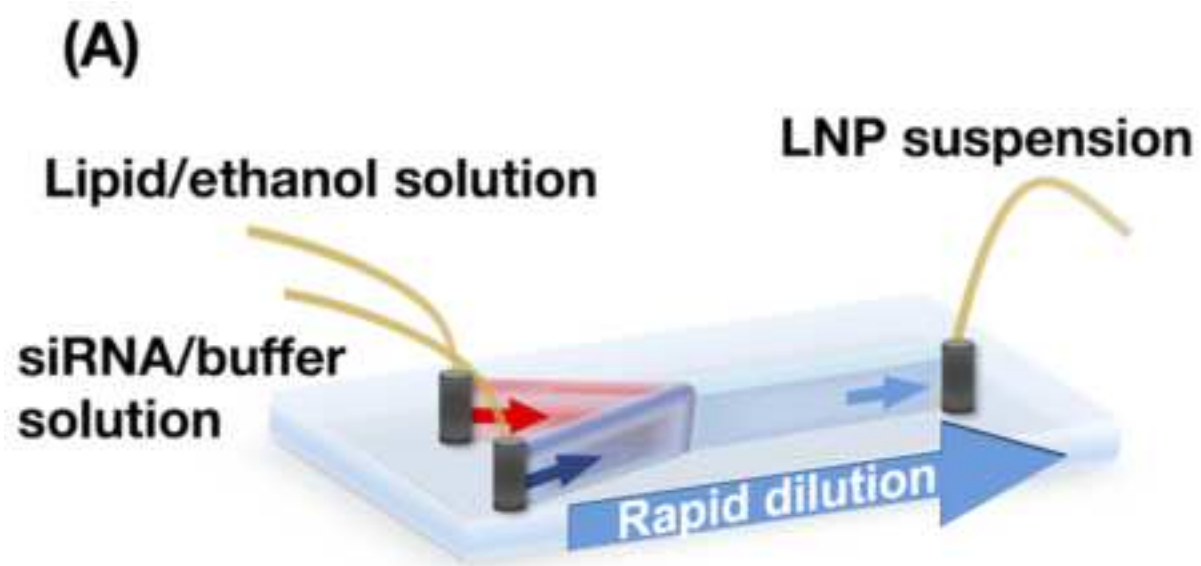
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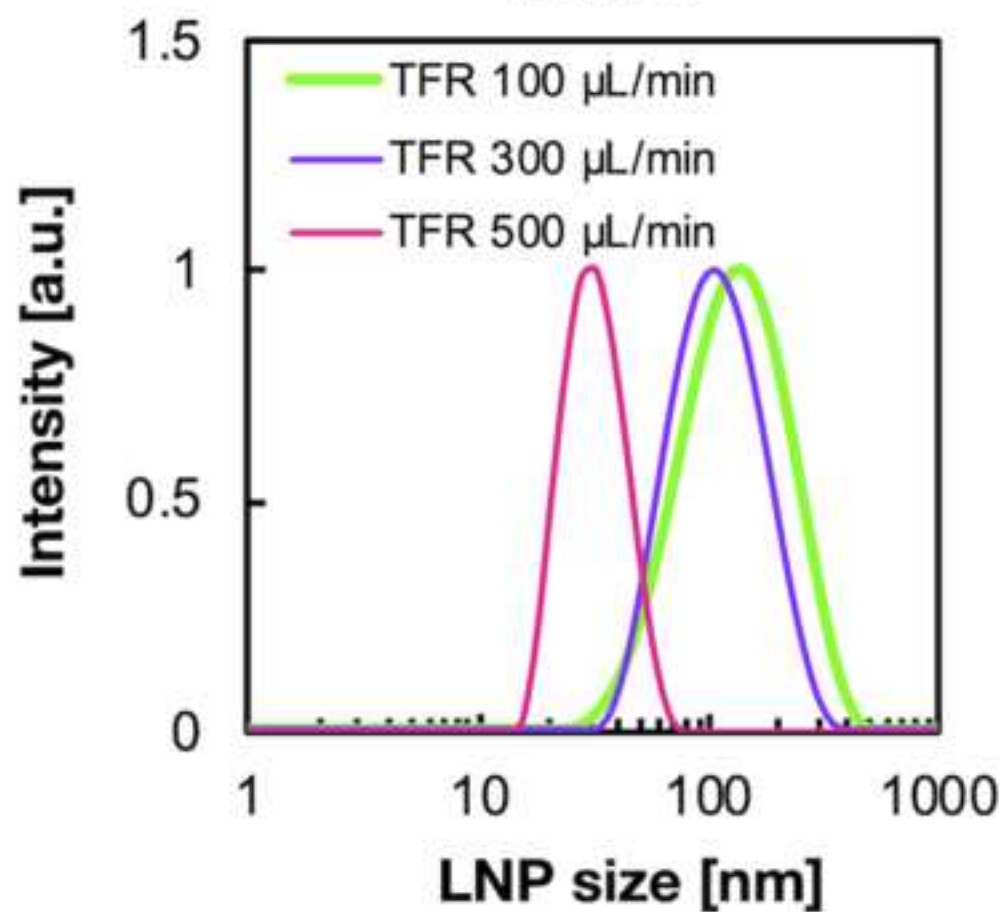
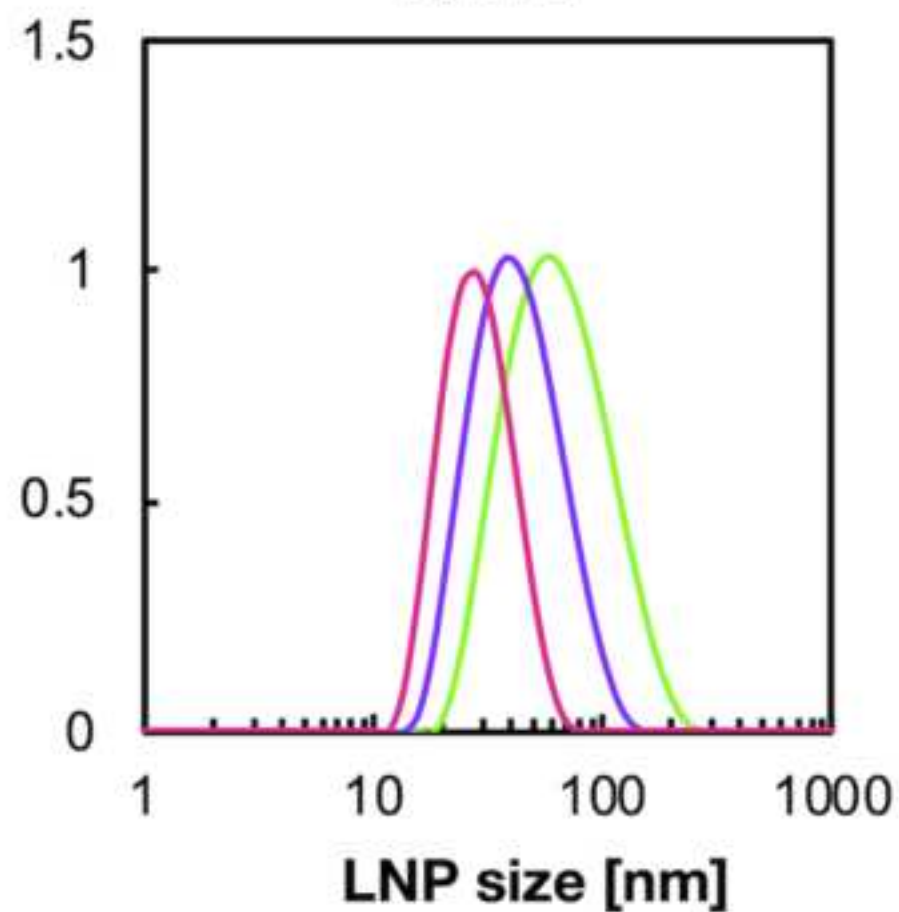
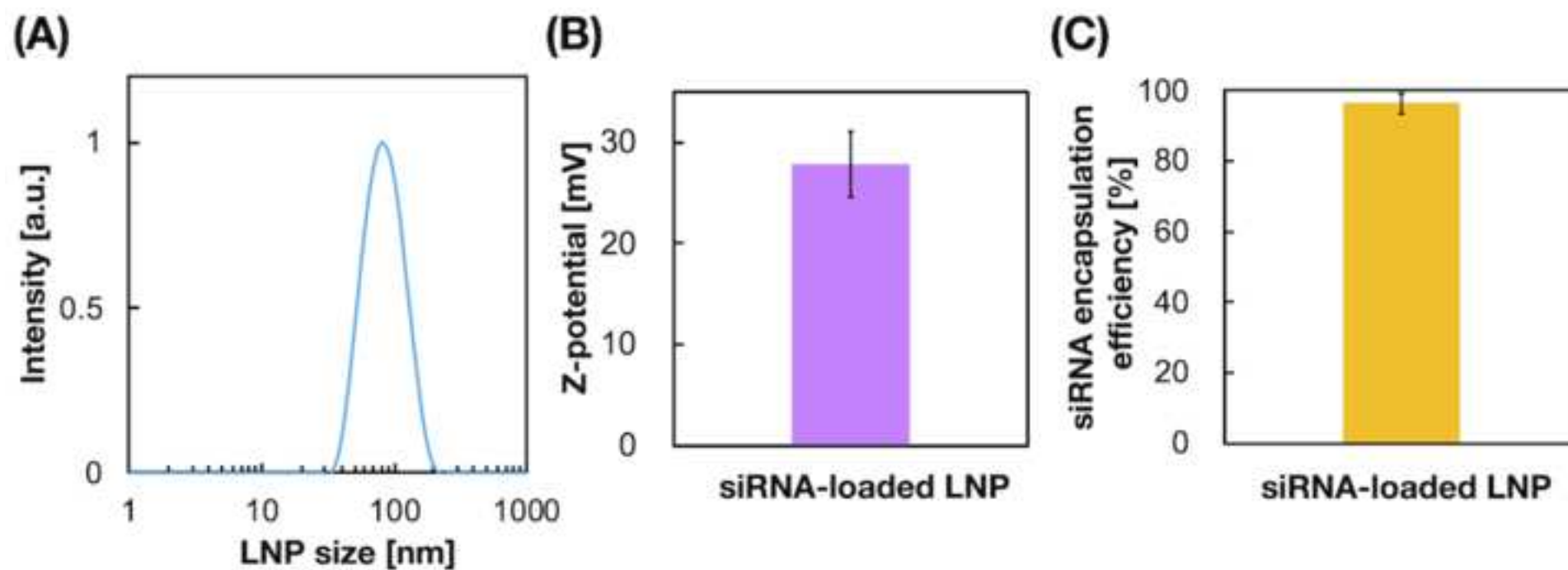
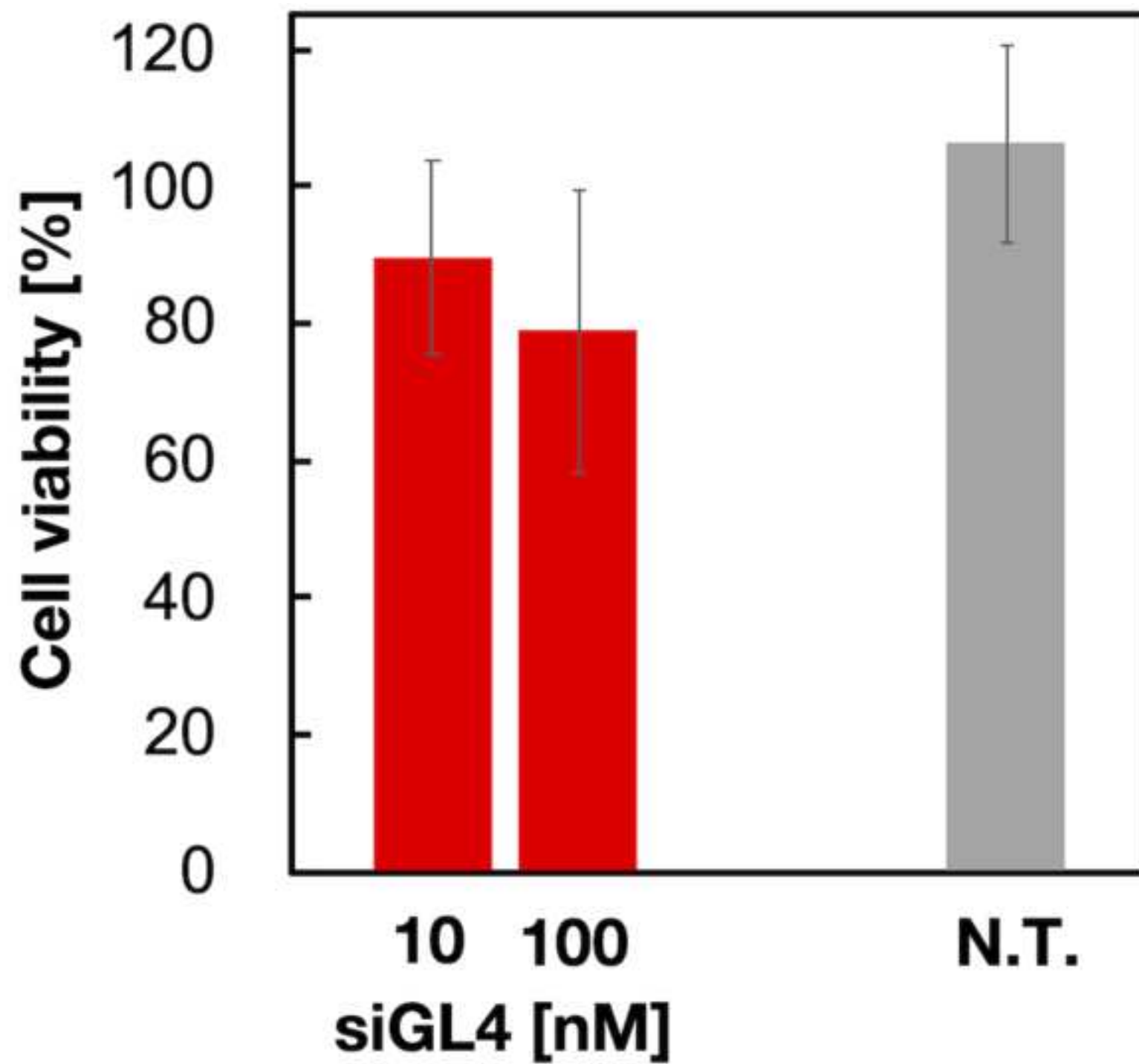
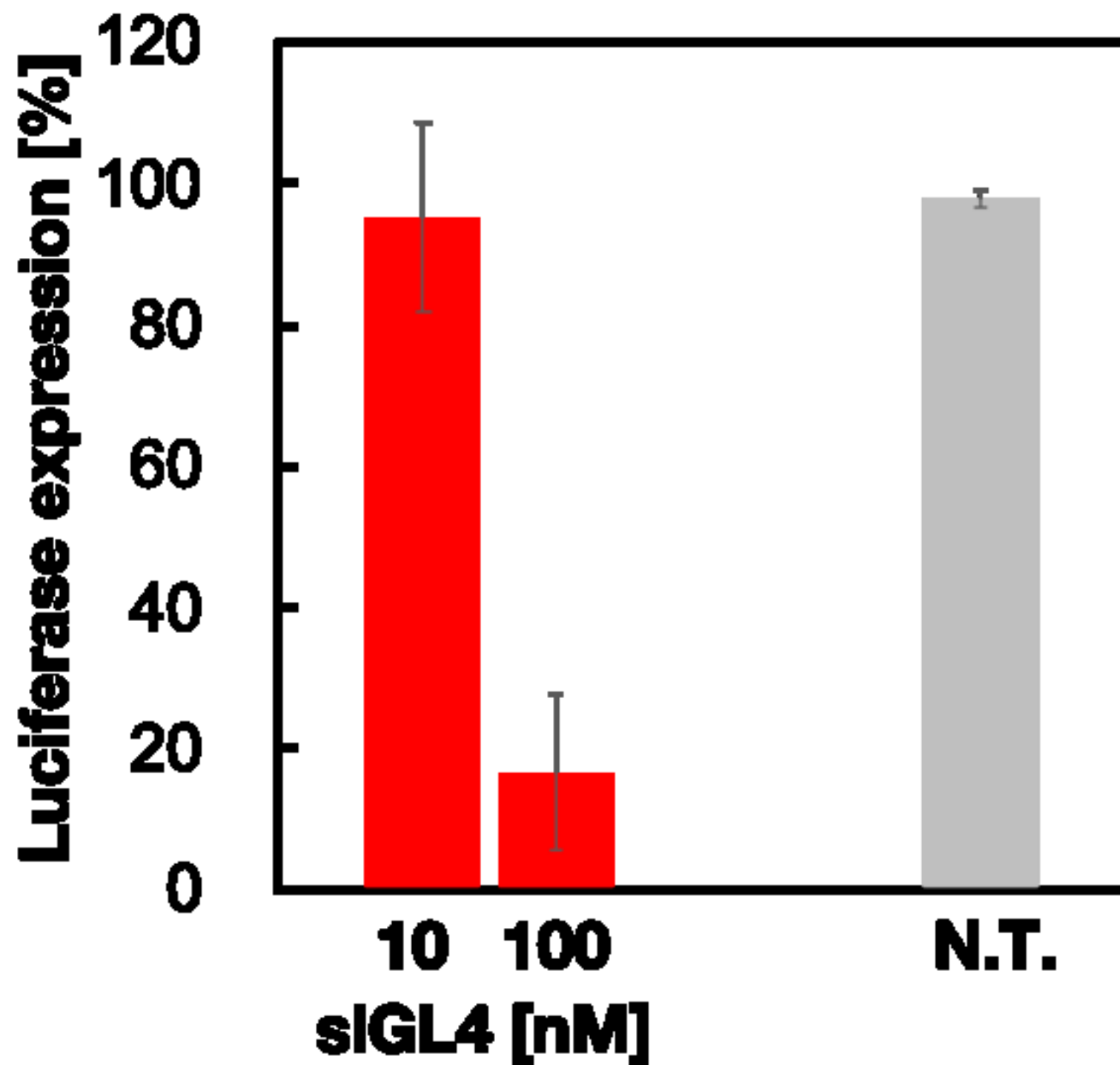
**(A)****FRR 3****(B)****FRR 5**

Figure 3











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**Table of Materials**

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Dr. Amit Krishnan  
Editor  
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Title: "Production of siRNA-Loaded Lipid Nanoparticles Using a Microfluidic Device"

Authors: Masatoshi Maeki, Yuto Okada, Shuya Uno, Ayuka Niwa, Akihiko Ishida, Hirofumi Tani, and Manabu Tokeshi

Dear Dr. Krishnan:

We have submitted the revised version of our manuscript. The thoughtful comments and suggestions by the reviewers have been helpful during revision of our manuscript. We made a comprehensive attempt to address all the main points made by the referees and herein provide a "point-by-point response to the reviewers' comments".

All the changes in the manuscript are shown in red text both in this document and in the revised manuscript file. We believe that these corrections and revisions have sufficiently answered the comments raised by the referees and we hope that the revised version of the manuscript is now acceptable for publication in *JoVE*.

Sincerely yours,

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## Point-by-point response to the comments raised by the reviewers

### Reviewer 1

#### General comment

There is no value in presenting graph with a single column. How many repeats were used?

**Response:** We have revised the graphs with error bars. Each experiment was repeated three times and calculated the average value and standard deviation.

**Changes:** (Caption of Figures 3-5): Data are represented as the mean + SD.  $n = 3$ .

#### Comment 1

The figures lack control- there is 1,20, 50 nM but no control.

**Response:** We appreciate your suggestion. We have revised the figures and added the result of non-treated samples.

**Changes:** (Caption of Figures 4-5): N.T.: Non-treated (D-PBS(-)).

#### Comment 2

Fig. 5 - no statistics.

**Response:** We have revised the graphs with error bars. Each experiment was repeated three times and calculated the average value and standard deviation.

**Changes:** (Caption of Figures 3-5): Data are represented as the mean + SD.  $n = 3$ .

#### Comment 3

The authors did not mention the particle size used. It is expected to compare couple of size as the authors mentioned the importance of particle size.

**Response:** We used 90 nm-sized siRNA-loaded LNPs as described in line 255. We have revised the sentence as shown in below.

**Changes:** (Lines 259-260): Cytotoxicity and the gene silencing activity of 90 nm-sized siRNA-loaded LNPs were evaluated as shown in **Figures 4 and 5**. The effect of LNP size on the gene silencing activity was reported previously<sup>6,13,17</sup>.

**Reviewer 2**  
**Comment**

Only a small point regarding the ribogreen assay. We have found triton affects the fluorescence, so we build a calibration curve for both with and without triton, so perhaps look at this. Also could you include how you measure yield or recovery, as we might have 90% loading but say only 40% of the LNPs back, so the conc would be wrong.

**Response:** We prepared two calibration curves for triton (+) and triton (-) samples. Therefore, the concentrations of siRNA were calculated from the appropriate calibration curve. This protocol is a standard protocol to determine the siRNA concentration (*Journal of Controlled Release*. **163** (3), 267-276, (2012)). We have revised the sentence as shown in below.

**Changes:** (Lines 167-168) Prepare the dilution series of siGL4 solution to make a calibration curve for triton (+) and triton (-) samples.

(Line 191) Calculate the siRNA encapsulation efficiency from the following equation<sup>19</sup>.

Ref. 19: Sato, Y. *et al.* A pH-sensitive cationic lipid facilitates the delivery of liposomal siRNA and gene silencing activity in vitro and in vivo. *Journal of Controlled Release*. **163** (3), 267-276, (2012).