Supplementary Information

Formulation of Lipid Nanoparticles Containing Ginsenoside Rg2 and Protopanaxadiol for the Highly Efficient Delivery of mRNA

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Supplementary methods

Analysis of the 5' and 3' UTR sequence of sub-genomic RNA of SARS-CoV-2. For the sequencing analysis, first total RNAs including all virus sub-genomic RNAs extracted from Vero cells infected with SARS-CoV-2, performed at the College of Veterinary Medicine of Chungnam National University (Daejeon, South Korea).

cDNA was generated through reverse transcription (RT) reaction on total RNA using oligo-dT (20-mer, Invitrogen, Massachusetts, USA). The reaction mixture (25 μL) contained 1.0 μg of total RNA, 50 μM of oligo-dT, and 10 mM dNTP (Invitrogen, Massachusetts, USA). Initially, an annealing reaction was carried out at 65°C for 5 min, followed by the addition of 5 × reaction buffer (Invitrogen), 25 mM $MgCl_2$ (Invitrogen), 0.1 M dithiothreitol (Invitrogen), Recombinant Ribonuclease Inhibitor, RNaseOut (Invitrogen), and SuperScript III RT (Invitrogen). The RT reaction proceeded for 50 min at 55 ℃, 5 min at 85 ℃, and 20 min at 37 ℃. Subsequently, the synthesized single-stranded cDNA underwent PCR to amplify sub-genomic RNA encoding nucleocapsid of SARS-Cov2, including the sequence of the 5'-UTR and 3' -UTR. For sequence analysis, two regions including 5' UTR and 3'UTR sequences were amplified by PCR. For the 5' UTR region, a 20 μL PCR mixture contained 1.0 ng of synthesized cDNA, 500 nM of the forward primer (Le-F) specific to the 5' end of the leader UTR sequence, 500 nM of the reverse primer (5U-R) specific to the 3' terminal region of the N gene of SARS-CoV-2, and 10 μL of 2 × *pfu* Master Mix (Biofact, Daejeon, South Korea). For the 3' UTR, 20 μL reaction mixture contained 500 nM each of forward (3U-F) and reverse (3U-R) primers specific for the 3'-terminal region sequence of SARS-CoV-2 was included in a 20 μL reaction mixture. PCR conditions were as follows: 1 cycle of 95 ℃ for 2 min, followed by 30 cycles of 95 ℃ for 20 s, 60 ℃ for 40 s, and 72 ℃ for 1 min, with a final extension at 72 ℃ for 5 min. The PCR products were then ligated into pTOP Blunt V2 vector (Enzynomics,

Daejeon, South Korea) and transformed into *Escherichia coli* DH5α (Enzynomics, Daejeon, South Korea). Post-transformation, bacterial cells were plated on 1.5% solid agar medium (Sigma Aldrich, USA) supplemented with ampicillin (50 μg/ml, Sigma Aldrich, USA) and incubated at 37 ℃ for 8 hrs. Plasmid DNA was purified using the HiGene™ Plasmid Mini Prep Kit (Biofact, Daejeon, South Korea). The 5' UTR and 3' UTR sequences were analyzed by Sanger sequencing (Biofact, Daejeon, South Korea). The PCR primer sequences are provided in Table S1.

DNA template construction for in vitro transcription of *EGFP and Luc* mRNA. To produce the *in vitro* transcription (IVT) template, all four fragments were prepared: the 5' UTR with a T7 promoter sequence at the 5' end, the *EGFP* or *Luc* gene fragment with 20 or 30 bp overlaps at both ends (3' end of the 5' UTR and 5' end of 3' UTR), and the 3' UTR (Fig. S1). The 5' UTR fragment, containing the T7 promoter sequence (5'-TAATACGACTCACTATAGGG-3') at its 5' end, was amplified by PCR using 500 nM each of the forward T7-F primer with a 24 bp overlap with the 5' end of the leader UTR sequence and the reverse 5U-R primer. To prepare the *EGFP* or *Luc* gene fragments with 20-30 bp overlaps at the 3' end of the 5' UTR and the 5' end of the 3' UTR, PCR was conducted using either pEGFP-N1 for EGFP (ClonTech, CA, UAS) or pGL3-basic (Promega, IW, USA) plasmid DNA as templates, with 500 nM each of forward (EGFP-F or Luc-F) and reverse (EGFP-R, Luc-R) primers. The 3' UTR was also amplified using 500 nM each of forward 3U-F and reverse 3U-R primers. The expected size of each PCR product was confirmed by agarose gel electrophoresis, and the PCR products were purified using the PCR Purification kit (Biofact, Daejeon, South Korea).

To generate templates for IVT, multiple overlap extension PCR was performed with slight modifications (Fig. S1). A 20 μL of PCR mixture included 50 ng of each fragment, 500

nM of both the forward T7-F primer and the reverse IVT-R primer featuring a 24 bp overlap with the 3' end of the 3' UTR, incorporating a 100-mer polyA at its 5' terminus, and 10 μL of 2 × *pfu* Master Mix (Biofact, Daejeon, South Korea). Thermal cycling conditions were as follows: 1 cycle of 95 ℃ for 2 min, followed by 30 cycles of 95 ℃ for 20 s, 60 ℃ for 40 s and 72 ℃ for 1 min. PCR primer sequences are presented in Table S1. The expected size of the PCR product was confirmed by agarose gel electrophoresis. The PCR product was cloned into the pTOP Blunt V2 vector, and the sequences were verified by Sanger sequencing.

For IVT of mRNA, the IVT template was amplified in a 20 μL of PCR mixture containing 1.0 ng of the plasmid harboring the IVT template, 500 nM each of the forward primer (IVT-F) specific to the plasmid vector sequence located in front of the T7 promoter sequence, and the reverse primer (IVT-R). The amplified PCR product was purified using the PCR Purification kit (Biofact, Daejeon, South Korea) and used as the IVT template. The PCR primer sequences are provided in Table S1.

Table S1. The primer sequences used in this study

Primers	Sequences (5' to 3')
Le-F	ATTAAAGGTTTATACCTTCCCAGGTAACAA
5U-R	TCTTCCTTGCCATGT TGAGT
3U-F	CAATCTTTAATCAGTGTGTAACATTAGG
3U-R	GTCATTCTCCTAAGAAGCTATTAAAATCAC
T7-F[a]	TAATACGACTCACTATAGGG*ATTAAAGGTTTATACCTTCCCAGG*
EGFP-F[a]	*CTCTAAACGAACAAACTAAA*ATGGTGAGCAAGGGCGAGGA
EGFP-R[a]	*TACACACTGATTAAAGATTG*TTACTTGTACAGCTCGTCCA
Luc-F[a]	*TAGATCTGTTCTCTAAACGAACAAACTAAA* ATGGAAGACGCCAAAAACATAAAGAAAG
Luc-R[a]	*TCCCTAATGTTACACACTGATTAAAGATTG*CTACACGGCGATCTTTCCG C
IVT-F	TCCACTAGTAACGGCCGCCA
IVT-R	PolyA 100-mer-GTCATTCTCCTAAGAAGCTATTAA

[a]Overlap sequences are indicated by italic letters.

Production of *EGFP* or *Luc* mRNA by IVT. IVT was performed using the HiScribe T7 mRNA Kit with CleanCap Reagent AG (New England BioLabs) following the manufacturer's instructions, with careful attention to maintaining sterility and ensuring all equipment and reagents were RNase-free. Briefly, 1 μg of DNA template was mixed with 2 μl of 10 × T7 CleanCap Reagent AG Reaction Buffer, 2 μL of ATP (60 mM), 2 μL of CTP (50 mM), 2 μL of GTP (50 mM), 2 μL UTP (50 mM), 2 μL of 3'O Me CleanCap Reagent AG (40 mM), and 2 μL T7 RNA Polymerase Mix. The reaction was then incubated for 16 hrs at 37 ℃. Purification of the mRNA transcripts was performed by DNase I digestion and LiCl-ethanol

precipitation, and the transcripts were dissolved in 150 μL of RNase free deionized water. The size of the *EGFP* mRNA and *Luc* mRNA was confirmed by gel electrophoresis and quantified using a Nanodrop spectrophotometer.

Supplementary images

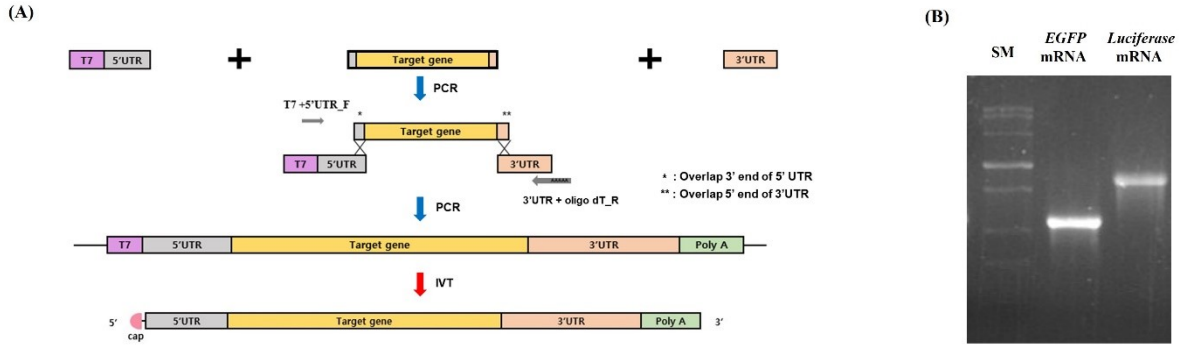

Fig S1. (A) Schematic illustration of production of *EGFP* mRNA and *Luc* mRNA by using modification of 5'UTR and 3'UTR sequences derived from the mRNA structure of SARS-CoV-2 via PCR and IVT process, and (B) Gel electrophoresis result of *EGFP* mRNA and *Luc* mRNA.

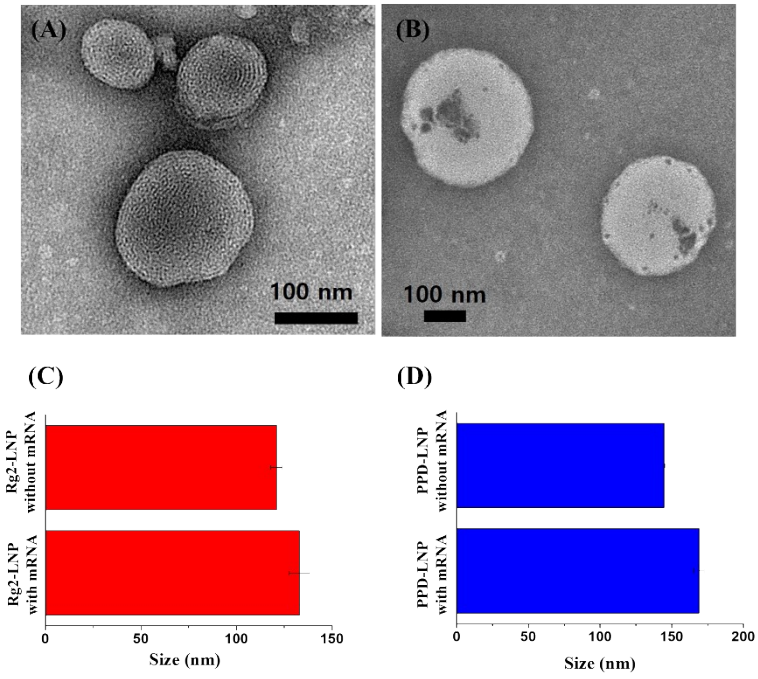

Fig S2. TEM images of (A) *EGFP* mRNA-Rg2-LNP and (B) *EGFP* mRNA-PPD-LNP, and size distribution analysis of (C) Rg2-LNP without/with mRNA and (D) PPD-LNP without/with mRNA via DLS measurement

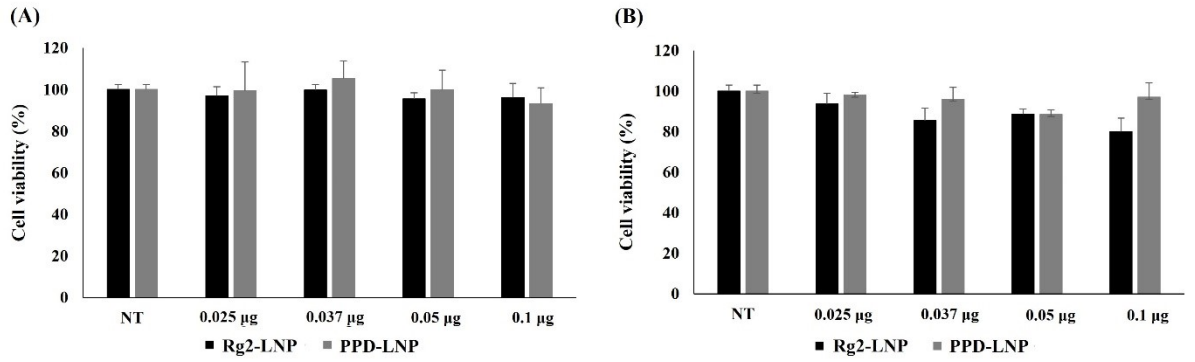

Fig S3. Cytotoxicity profiles depending on the concentration of LNPs. (A) A549 cell and (B) HeLa cell.

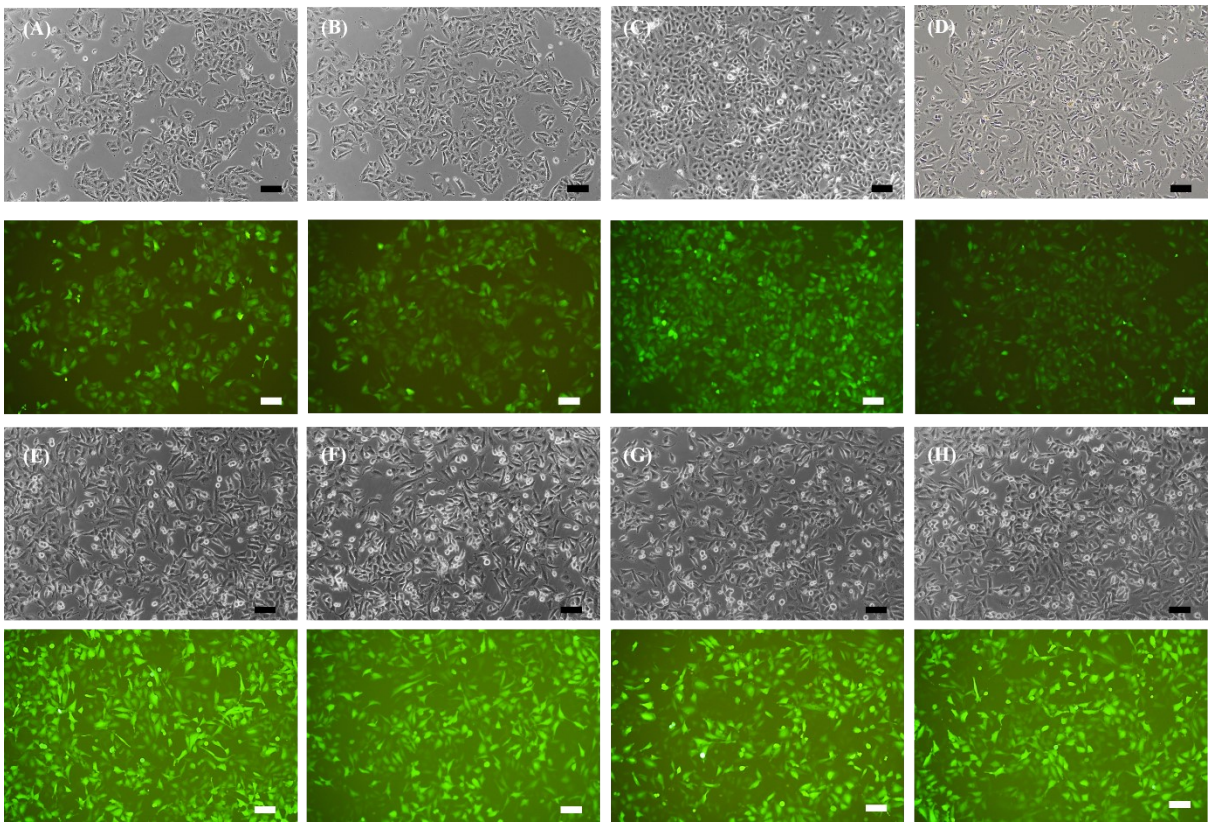

Fig S4. *GFP* mRNA delivery and GFP expression test depending on the molar ratios of Rg2 or PPD in LNP formulations in A549 cell ((A) to (D)) and HeLa cell ((E) to (H)). (A) and (E) 50 : 10 : 1.5 : 39, (B) and (F) 50: 10: 1.5 : 78 in Rg2-LNP, as well as (C) and (G) 50 : 10 : 1.5 : 22.9, (D) and (H) 50 : 10 : 1.5 : 45.8 for PPD-LNP (scale bar: 100 μm).

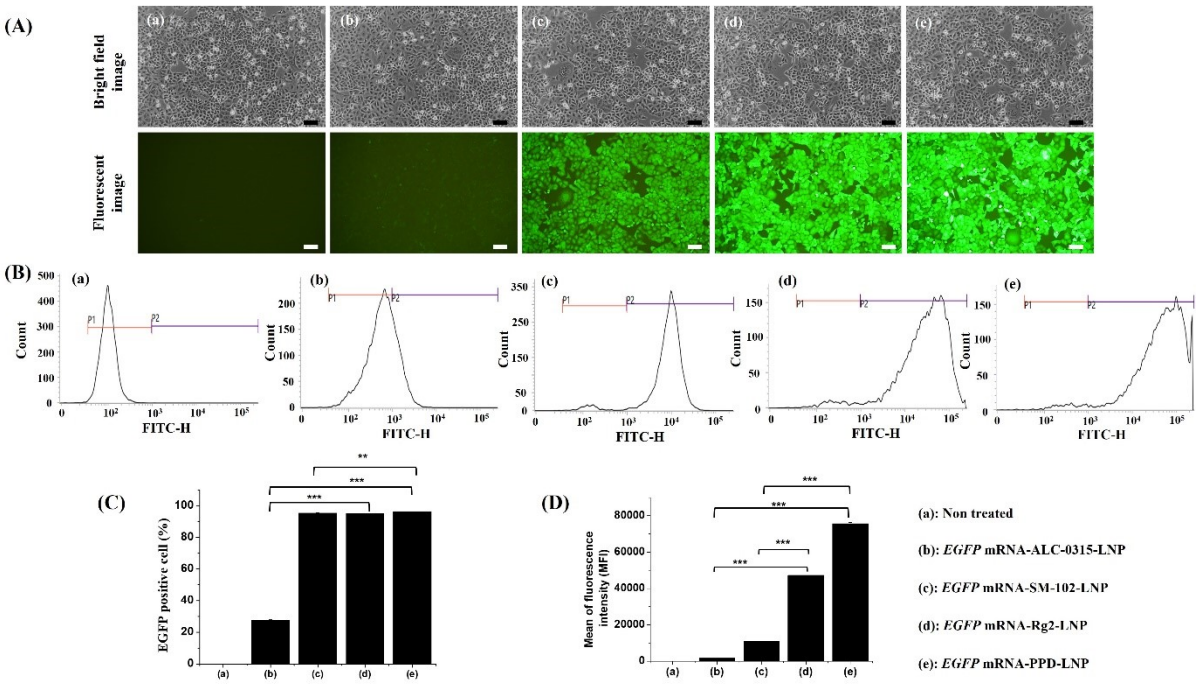

Fig. S5 Comparison of GFP expression efficiency in A549 between LNPs produced by commercial-LNPs, Rg2-LNP and PPD-LNP. (A) Optical microscope images (B) Histogram, (C) percentage of the EGFP-positive cells and (D) MFI depending on the LNP condition; (a) non-treated, (b) *GFP* mRNA-ALC-0315-LNP, (c) *GFP* mRNA-SM-102-LNP, (d) *GFP* mRNA-Rg2-LNP and (e) *GFP* mRNA-PPD-LNP. Statistical difference was defined as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (scale bar: 100 μm)

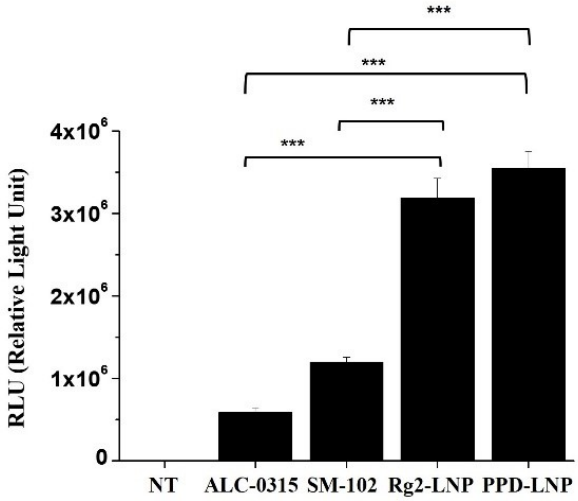

Fig. S6. Luc expression profile of ALC-0315-LNP, SM-102-LNP, Rg2-LNP and PPD-LNP with *Luc* mRNA in A549 cell. Statistical difference was defined as *p < 0.05, **p < 0.01, ***p < 0.001

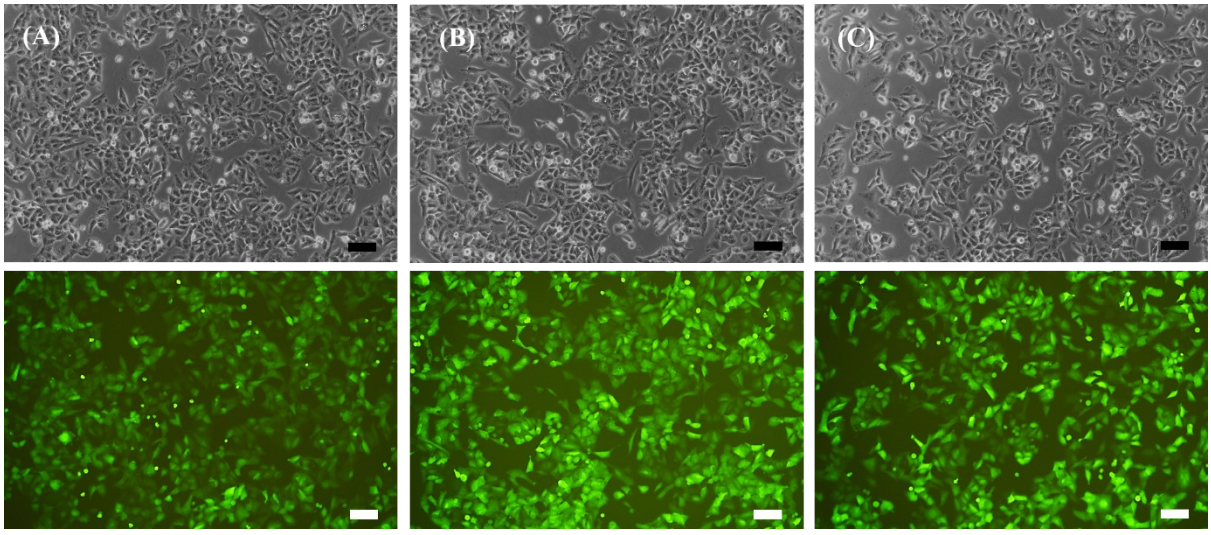

Fig. S7. Observation of GFP expression in A549 cells after *GFP* mRNA delivery via (A) SM-102/DOPE/cholesterol/PEG lipid based LNP (modified-SM-102 LNP), (B) SM-102/DOPE/Rg2/PEG lipid based LNP (Rg2-LNP) and (C) SM-102/DOPE/PPD/PEG lipid based LNP (PPD-LNP) (scale bar: 100 μm).

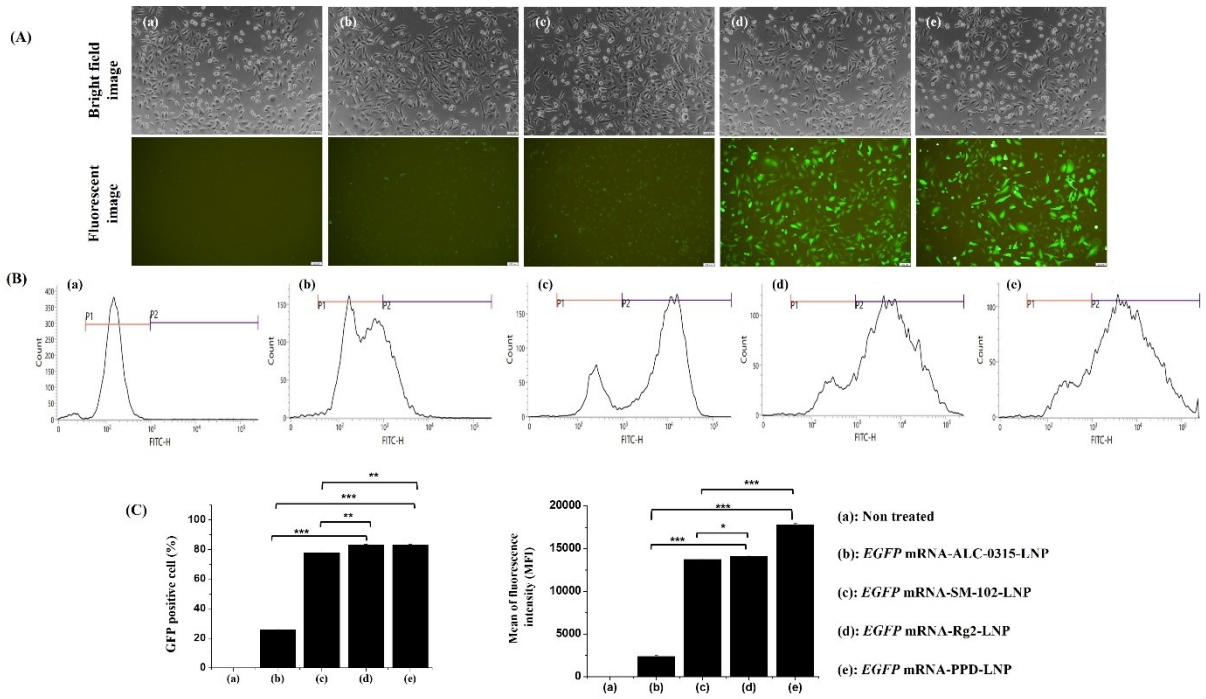

Fig. S8 Comparison of expression efficiency in HeLa between LNP produced by commercial-LNPs, Rg2-LNP and PPD-LNP. (A) Optical microscope images (B) Histogram, (C) percentage of the EGFP-positive cells and (D) MFI depending on the LNP condition. (a) non-treated, (b) *GFP* mRNA-ALC-0315-LNP, (c) *GFP* mRNA-SM-102-LNP, (d) *GFP* mRNA-Rg2-LNP and (e) *GFP* mRNA-PPD-LNP; Statistical difference was defined as *p < 0.05, **p < 0.01, ***p < 0.001. (scale bar: 100 μm)

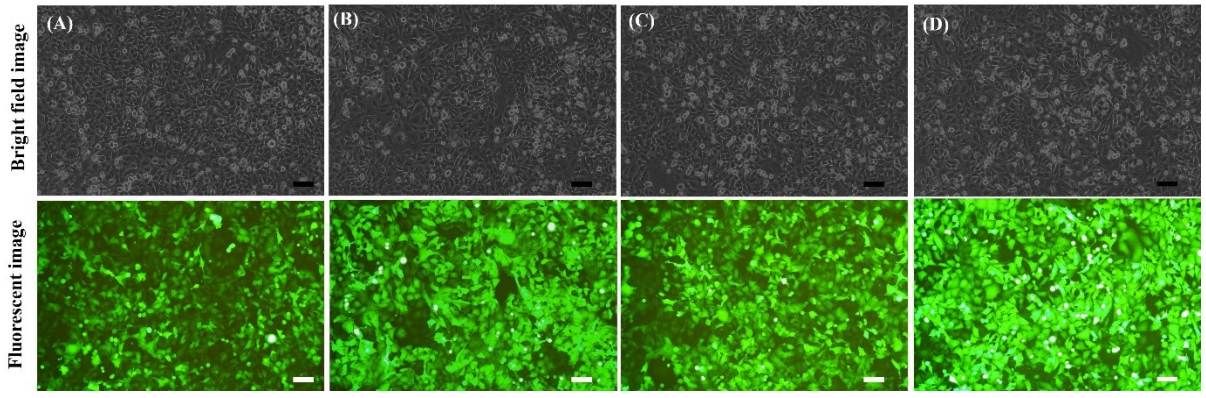

Fig. S9. Monitoring of EGFP expression efficiency depending on the molar ratio of PEG lipid in LNP formulation (molar ratio = ionizable lipid : phospholipid : PEG lipid : Rg2 or PPD). (A) 50: 10: 1.5 : 39, (B) 50 : 10 : 0.75 : 39 molar ratio of Rg2-LNP with *EGFP* mRNA as well as (C) 50 : 10 : 1.5 : 22.9, (D) 50 : 10 : 0.75 : 22.9 molar ratio of Rg2-LNP with *EGFP* mRNA. (after treatment in 48 hrs, scale bar: 100 μm).

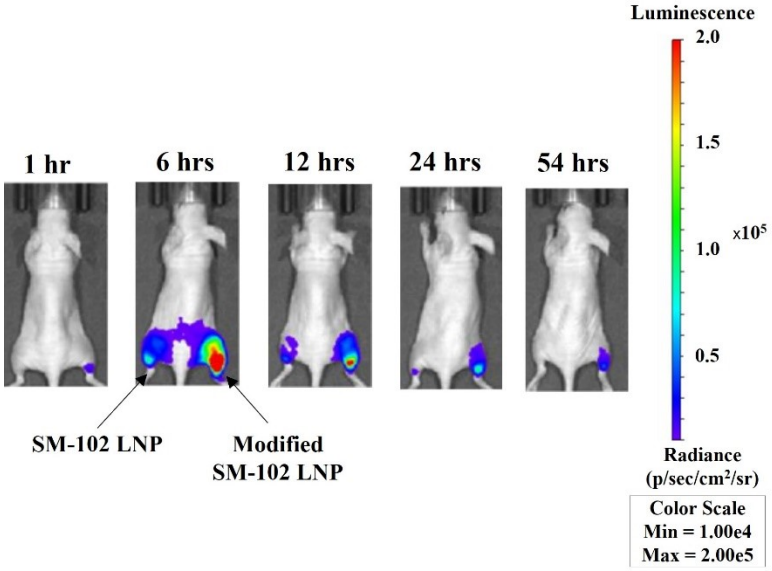

Fig. S10. Observation of *in vivo* expression of Luc in hairless mouse using *Luc* mRNA-SM-102-LNP (left) and *Luc* mRNA-modified SM-102-LNP (right) as positive control test.

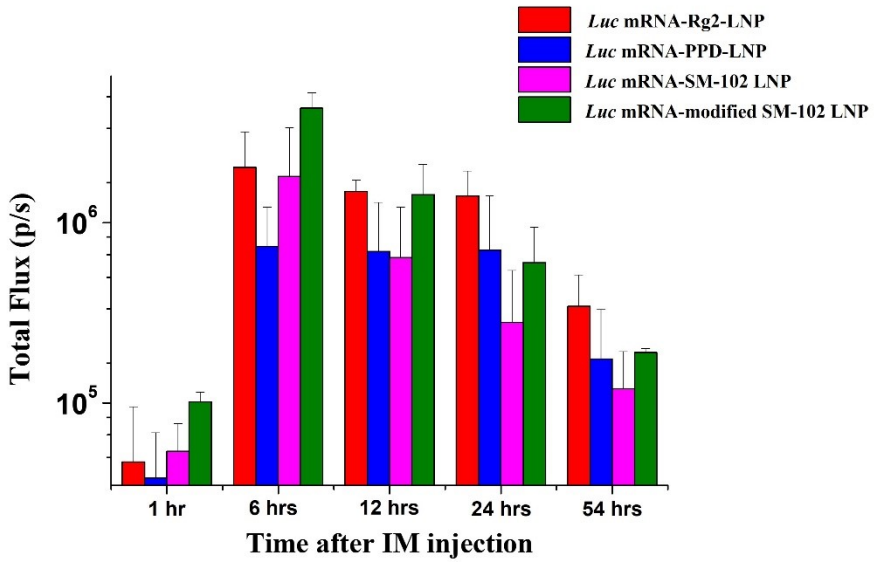

Fig. S11. Measurements of Luc activities at various time points after *Luc* mRNA-LNP injection