Supplemental Information

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1. Issues with TransIT for transfection of PRINT protein particles

*Trans*IT®-mRNA Transfection Kit is composed of a TransIT reagent and a boost. In order to achieve the optimum particle transfection with TransIT, we tested different TransIT reagent: boost : particle ratios and the results (Figure S1) indicated that at a constant particle concentrations, higher doses of TransIT and boost decreased protein expression. This might be due to some inhibitory effects on Vero cells from TransIT.

At a constant ratio of TransIT reagent:boost= 2μL:1μL per well, higher particle concentrations showed reduced protein expression. Flow cytometry was performed to quantify the percentage of cells that internalized the TransIT coated particles. As shown in Figure S2, the Vero cells showed less particle internalization as the particle concentration increased. Further study using fluorescence microscopy (Figure S3) showed that at high particle concentration, particles showed severe aggregation, which may explain reduced cell uptake of the particles. From these results, it can be concluded that TransIT can only transfect the RNA replicon loaded protein particles with a limited efficacy.

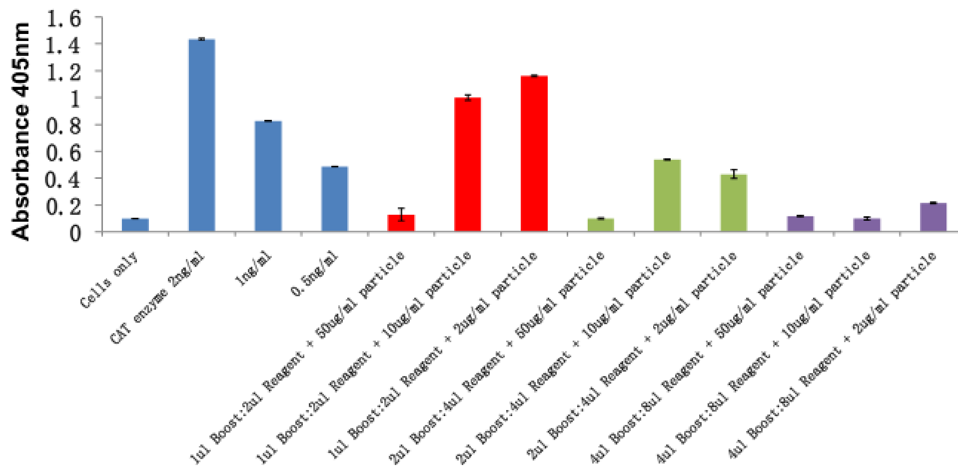


Figure S1. CAT protein expression resulting from transfection of RNA replicon-containing BSA PRINT particles coated with TransIT. Error bars represent standard deviation calculated from three wells.

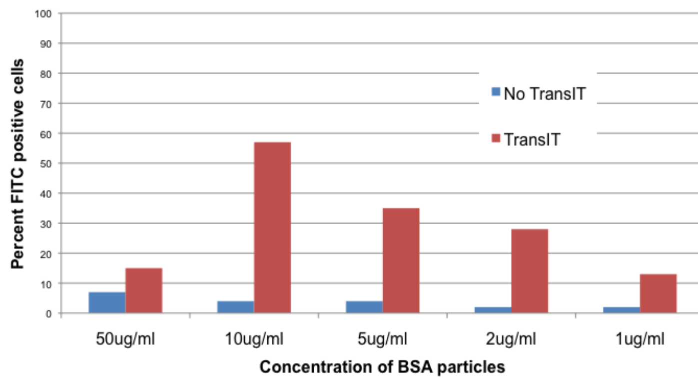


Figure S2. Particle internalization characterized by flow cytometry. Red: protein particles coated with TransIT. Blue: protein particles only.

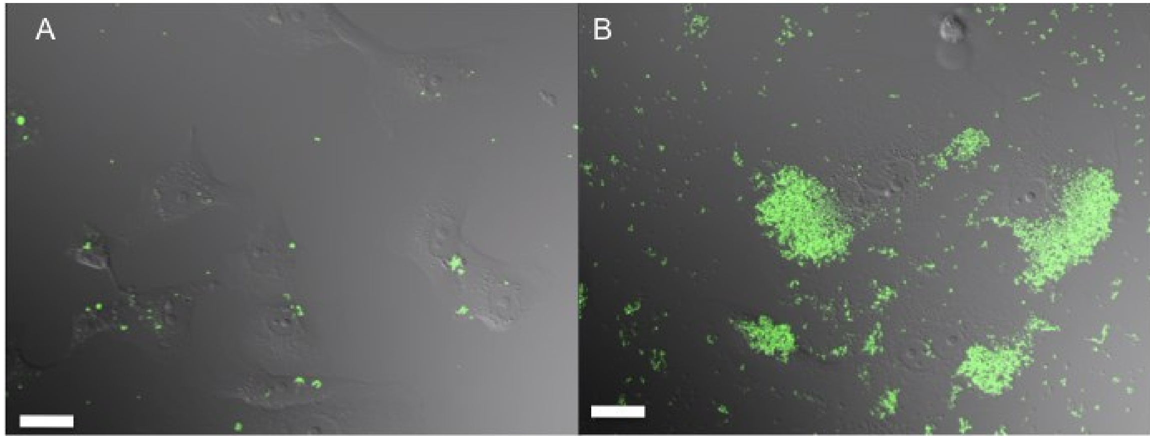


Figure S3. Particle internalization after 24 h, characterized by Confocal imaging (A) 2 μg/mL, (B) 50 μg/mL, green: Alexa fluoro 488 labeled particles. Scale bars represent 20 μm.

2. Fabrication and stabilization of RNA replicon incorporated PRINT Particle

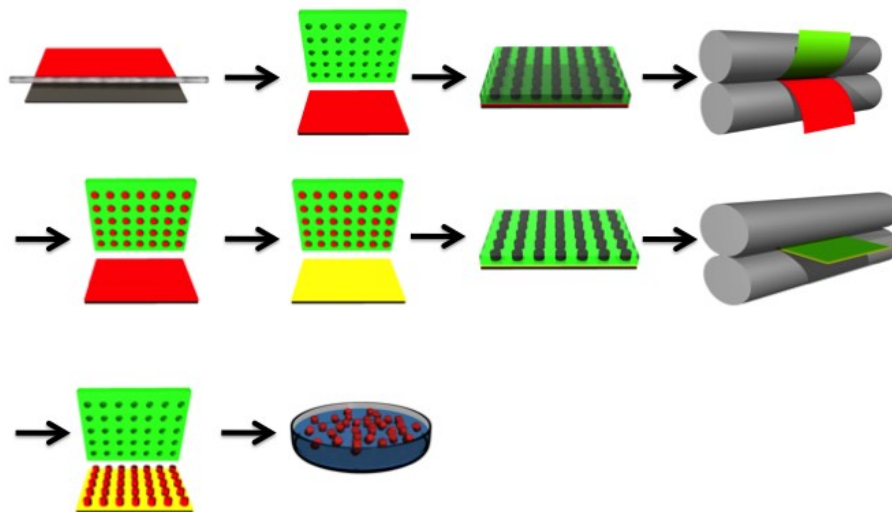


Figure S4. The PRINT Process. BSA, lactose, glycerol and RNA replicon were mixed in water to create a solution. A wet film of this solution was cast on a plastic sheet with a myer rod. Water is removed with assistance from a heat gun and a solid film is formed

(red). A PRINT mold (green) is put on top of the film. The mold and the film were then passed through a heated pressured nip (grey) and split. The PRINT mold is then brought in contact with a sacrificial adhesive layer (yellow) and passed through the heated nip again. After the mold cools down to room temperature, the mold and the sacrificial layer are separated gently and particles are now with the sacrificial layer, which is then dissolved to free the particles.

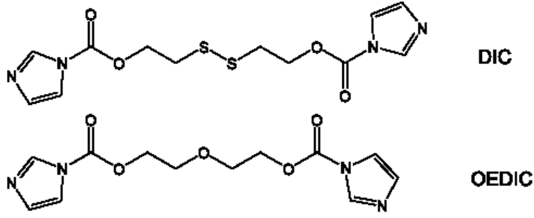
Table S1 Particle composition

	Charged Composition [a] (wt%)	Final Composition [b] (wt%)
BSA	37.0	81.5 ± 0.2
Lactose	37.0	10.3 ± 3.1
Glycerol	25.0	6.7 ± 2.8
RNA Replicon	1.0	1.5 ± 0.1

[a] The weight percentage of BSA, lactose, glycerol and RNA charged into the preparticle solution. [b] Final particle composition after harvest and purification step. The errors stand for standard deviation calculated from three experiments.

3. Characterization of cross-linked BSA particles

a)



b)

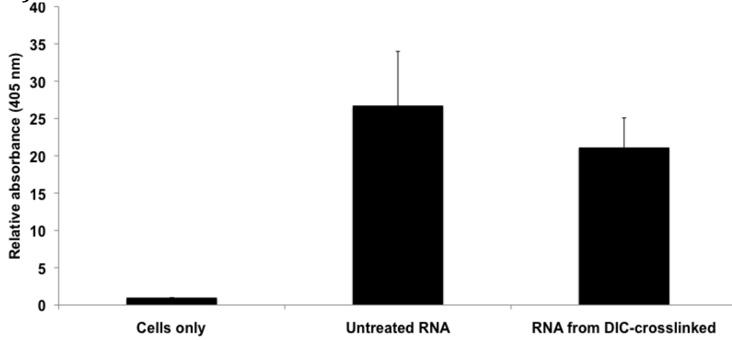


Figure S5. a) Structure of cross-linkers: dithio-bis(ethyl 1 H-imidazole-1-carboxylate) (DIC) and 2,2'-oxybis(ethane-2,1-diyl) bis(1H-imidazole-1-carboxylate) (OEDIC); b) RNA replicon integrity after crosslinking reaction evaluated by CAT ELISA. The absorbance from un-treated cells (cells only) was defined as 1. Error bars= mean + SD.

The CAT RNA replicon was incubated with IPA and DIC/IPA (DIC concentration 1.5 mg/mL) at a concentration of 300 µg/mL for 24h at 40 °C. The IPA was removed through evaporation under vacuum and the RNA was dissolved in water and purified through a chloroform-Qiazol method. The integrity of the RNA replicon was tested by dosing the RNA with TransIT to the Vero cells and measuring CAT protein expression.

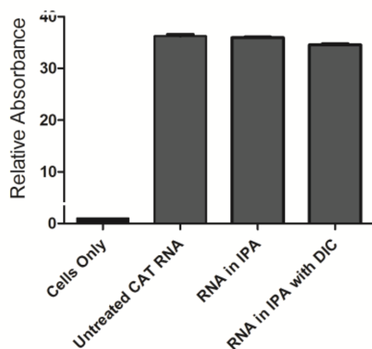


Figure S6. RNA replicon integrity evaluated by CAT ELISA 1) cells only; 2) untreated CAT RNA control 100 ng/mL; 3) CAT RNA incubated with IPA for 24h at 40 °C, 100 ng/mL; 4) CAT RNA incubated with IPA and DIC (1.5mg/mL) for 24h at 40 °C, 100 ng/mL. The absorbance from un-treated cells (cells only) was defined as 1.

In order to further investigate the release of RNA from cross-linked particles, two RNA molecules (a double-stranded 21-bp RNA and a double-stranded ~400-bp RNA) were encapsulated in the BSA particles and cross-linked under the same conditions. The particles loaded with the 21-bp RNA were incubated with PBS at room temperature and pelleted through centrifugation. The supernatant and the particles were characterized using Argarose gel electrophoresis (Figure S7). The results showed that almost all of the 21-bp RNA leached out the particles during incubation and very minimal are left in the particles. In addition, the bands representing the RNA in the supernatant and the RNA found in the uncross-linked particles have similar intensity (quantified using software ImageQuant TL). For the 400-bp RNA (data not shown), the RNA extracted out of the DTT degraded particles displayed a bright band similar to that associated with un-crosslinked particles and only small amount of RNA leached out the particles during incubation in PBS. These results indicated that it is possible to fully recover RNA from

the cross-linked particles for low molecular weight RNAs without damaging their integrity. For the ~9kb RNA replicon, maybe due to its large size, only a small portion of it can be recovered.

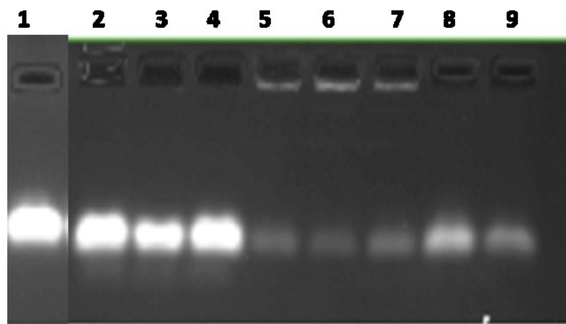


Figure S7: Agarose gel of 21-bp RNA before and after particle crosslinking:

lane 1: RNA extracted out of 37.5 μg of BSA particles before crosslinking reaction,

2: RNA in the supernatant after 5min incubation (37.5μg of particles), 3: RNA in the supernatant after 20min incubation (37.5μg of particles), 4: RNA in the supernatant after 100min incubation (37.5μg of particles), 5: RNA left in 37.5μg of particles after 5min incubation, 6: RNA left in 37.5μg of particles after 20min incubation,7: RNA left in 37.5μg of particles after 100 min incubation, 8: untreated RNA 100 ng, 9: untreated RNA 50 ng.

Methods:

Evaluation of RNA replicon activity through CAT expression:

Typically, 2×10^4 Vero cells were plated into 24 well tissue cultured treated plates 18-24 h prior to assay. Vero cells were transfected with CAT RNA replicon utilizing the TransIT® mRNA transfection kit (Mirus Bio, Madison, WI) following the manufacturer's protocol. Cell lysates were prepared 48h post-transfection and CAT

ELISA (Roche, Indianapolis) analysis was carried out according to the manufacturer's instructions. The relative absorbance was calculated using following method:

$$Ar = \frac{Aa}{Ac}$$

Where Ar: the relative absorbance

Aa: the absorbance acquired by plate reader for samples dosed with RNA replicon or particles

Ac: the absorbance acquired by plate reader for untreated cells.

Analysis of CAT expression and immunofluorescence microscopy:

The analysis of CAT protein expression and imaging of CAT protein immunofluorescence were carried out following procedures described in [26].

4. **Investigation of the complexation between the DOTAP/DOPE and cross-linked RNA replicon loaded protein particles.**

To investigate the interaction in this complexation between the lipids and the cross-linked RNA replicon-loaded protein particles, LPP particles were incubated a number of different solutions or solvents. LPP particles were treated with 1×PBS, 10×PBS, NaCl (100 mM), Tween 20 (0.1% v/v), sodium dodecyl sulfate (SDS, 0.1%), and trifluoro ethanol (TFE) at 1 mg/mL for 30 min and washed three times through centrifugation. Table S2 shows the percentage of lipids (DOTAP and DOPE) that were removed during the treatment. The complexes were partially dissociated by Tween 20 and SDS due to hydrophobic competition. The Complexes were also partially disrupted by TFE, a good organic solvent for the lipids. In contrast, NaCl, 1×PBS and 10×PBS were ineffective in

dissociating the complexes. These results illustrate that hydrophobic interaction contributes to the lipid-particle complexation, and ionic interaction does not. Since SDS, Tween 20 and TFE only removed part of the lipids associated with the particles, we suspect that lipids are not only complexed to the surface of the cross-linked protein particles, but also penetrate to the interior of the particles which become porous due to the removal of processing plasticizers lactose and glycerol.

Table S2. Percentage of Lipids removed by solutions or solvent

Solution or Solvent	% of DOTAP removed [a]	% of DOPE removed [a]
NaCl	Not detectable	Not detectable
1×PBS	Not detectable	Not detectable
10×PBS	Not detectable	Not detectable
SDS	8% ± 1%	4% ± 0%
Tween 20	8% ± 5%	4% ± 3%
TFE	22% ± 5%	19% ± 3%

a. The error stands for standard deviation calculated from three experiments.

Table S3. Characterization of cross-linked BSA particles with and without DOTAP/DOPE lipids [a]

	Diameter, nm	PDI	ζ-Potential, mV [b]
Without lipids	1262	0.180	-15.4 ± 1.0
With lipids	1048	0.235	+29.3 ± 2.5

a. The particles charged with 1 wt% of CAT RNA replicon. The zeta potential was measured in 1mM KCl.

b. The error stands for standard deviation calculated from three experiments.

5. The 3D image of CAT protein and LPP particles inside Vero cells and microscopic analysis of positive cells

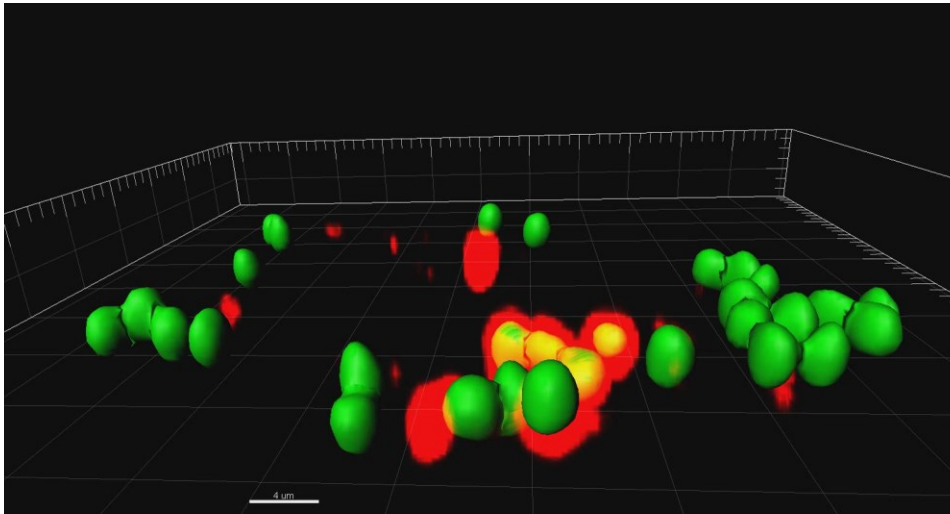


Figure S8. The 3D image of CAT protein and LPP particles inside Vero cells. The red represents the CAT protein and the green represents the particles. The scale bar represents 4μm.

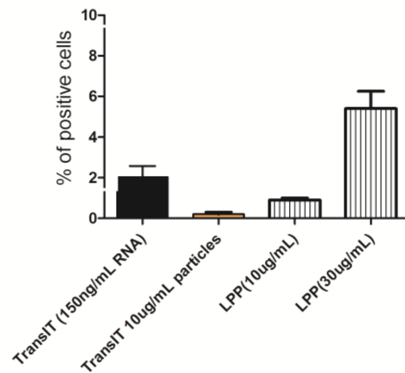


Figure S9. Microscopic analysis of positive cells. Number of cells expressing luciferase were imaged (Figure 6b) and percentage of positive cells were calculated. For every sample, three pictures were randomly taken with ~500 cells in the view.

6. **Comparison between DOTAP/DOPE only and LPP particles**

The efficacy of RNA replicon delivery using DOTAP/DOPE mixture alone was compared with PRINT particles mediated delivery. The LPP particle solution was prepared at 1 mg/mL of particle concentration (equal to 15 μg/mL of RNA replicon concentration). The RNA-lipid complexes were prepared at a constant RNA concentration (15 μg/mL), with different lipid concentrations ranging from 0.075 to 2.4 mg/mL. The results indicated that DOTAP/DOPE can deliver the RNA replicon most efficiently when prepared at a lipid concentration of 0.3~0.6 mg/mL (black dot bars). However, with similar amount of lipids (0.6mg/mL) complexed, the LPP particles achieved a luciferase protein expression 5 times higher than DOTAP/DOPE alone (orange square bars). This result demonstrated the value of using PRINT particles for delivery of RNA replicon.

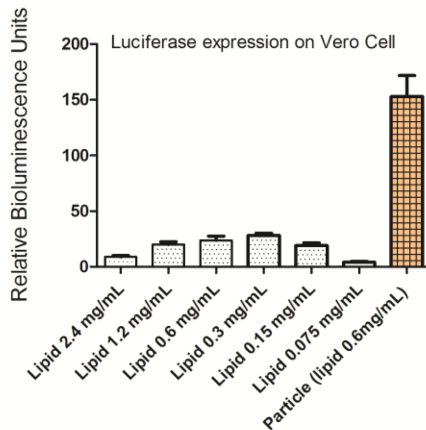


Figure S10: Relative bioluminescence generated by LPP particles and RNA-lipid complex on Vero cells. The luminescence generated by untreated cells is defined as 1. Black dots: Luciferase RNA replicon delivered by DOTAP/DOPE alone, orange square: LPP particles containing Luciferase RNA replicon. All samples dosed at a final RNA concentration of 450 ng/mL. Error bars= mean + SD.

7. Particle Protection of RNA replicon against RNase A

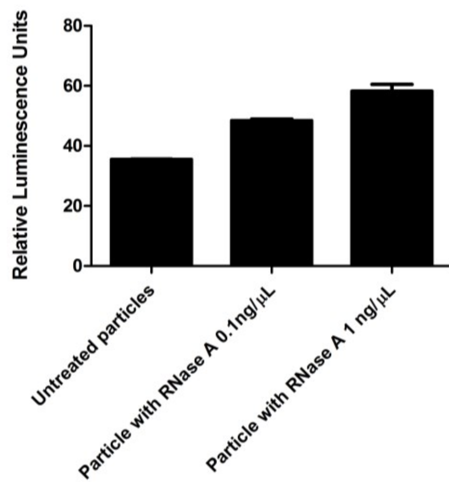


Figure S11. Integrity of RNA replicon against RNase A. The luciferase RNA replicon-loaded particles were incubated with RNase A at different concentrations for 1h at 37 °C followed by incubation with Vero cells for 4h. The particles were then removed and the cells were further incubated for 24h to allow luciferase to be expressed. Particle concentration is 30 μg/mL. Error bars= mean + SD.

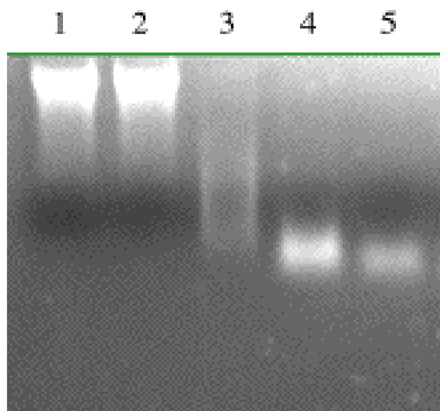


Figure S12. Free RNA degradation by RNase A.
Lane 1 and 2: untreated RNA replicon
Lane 3: RNA replicon incubated with 0.1 ng/μL RNase
Lane 4: RNA replicon incubated with 1 ng/μL RNase
Lane 5: RNA replicon incubated with 5 ng/μL RNase.

Methods:

The LPP particles containing luciferase RNA were dispersed in PBS containing 0, 0.1, and 1 ng/μL of RNase A at 1 mg/mL of particle concentration and incubated for 1h at 37 °C. The particles were then washed with water through centrifugation to remove unreacted RNase A. The particles were then dispersed in water with 5% dextrose and dosed to cells as previously described.