Supplementary Information for

Development of light-induced disruptive liposomes (LiDL) as a photoswitchable carrier for intracellular substance delivery

Taichi Tsuneishi[a], Keiichi Kojima[a,b], Fumika Kubota[c], Hideyoshi Harashima[c], Yuma Yamada[c] and Yuki Sudo[a,b*]

[a]Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, 700-8530, Japan.
[b]Faculty of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, 700-8530, Japan.
[c]Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, 060-0812, Japan.

*To whom correspondence should be addressed.
Yuki Sudo; Telephone: +81-86-251-7945, E-mail: sudo@okayama-u.ac.jp

Keywords: rhodopsin, liposome, proton pump, intracellular delivery

Table of Contents

1. Experimental details, materials and methods

Preparation of *Rm*XeR

Techniques used for gene preparation, protein expression and purification of histidine-tagged *Rm*XeR were performed as previously described.[1] In short, *E. coli* BL21 (DE3) cells harboring the plasmid were grown at 37 °C in LB medium supplemented with ampicillin (FUJIFILM Wako Pure Chemical Industries, Ltd., Japan; final concentration of 50 μg/mL). Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (FUJIFILM Wako Pure Chemical Industries, Ltd., Japan; final concentration of 1 mM) and all-*trans* retinal (Sigma-Aldrich, USA; final concentration of 10 μM). The cells were harvested by centrifugation (4,000 × *g* for 10 min) at 4 °C and were then resuspended in a buffer (50 mM Tris-HCl, pH 8.0) containing 1 M NaCl. They were then disrupted by sonication (UD-211, TOMY Seiko Co., Ltd., Japan; Output 7, Duty 50 (1 pulse / 0.5 sec)) in the same buffer. The crude cellular membranes were collected by ultracentrifugation (130,000 × *g* for 1 hr) at 4 °C and then solubilized with *n*-dodecyl-β-D-maltoside (DDM, Dojindo Laboratories, Japan; final concentration of 1.0% (w/v)). The solubilized fraction was purified with Ni^{2+} affinity (His Trap™ FF) (Cytiva, USA) and gel-filtration column chromatography (Superdex™ 200 increase 10/300 GL (Cytiva, USA)). During the purification, the sample solution was exchanged to a buffer containing 100 mM NaCl (pH 6.8-7.2) or 1 mM HEPES (pH 7.4) and 0.01% DDM (pH 7.4). Protein concentrations were adjusted to 3 mg/mL by ultrafiltration using an Amicon Ultra-15-30K filter (Merck KGaA, Germany).

Preparation of liposomes

Egg yolk phosphatidylcholine (EggPC) and cholesterol (Avanti Polar Lipids, Inc., USA) were mixed at a 7:3 molar ratio to produce pH-insensitive liposomes, while 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS) (Avanti Polar Lipids, Inc., USA) were mixed at a 7:3 molar ratio to produce pH-sensitive liposomes. The liposomes were prepared by the standard hydration method as described elsewhere.[2] In short, 1.25 μmol lipids were dissolved in 500 μL organic solvent containing ethanol and chloroform (FUJIFILM Wako Pure Chemical Industries, Ltd., Japan) at a 1:1 volume ratio. The lipid films were dried on the bottom of glass tubes (12 mm × 90 mm, P12-M (Nichiden Rika-glass Co., Ltd., Japan) by removing the organic solvent with nitrogen gas. To generate liposomes, 500 μL of a solution containing 100 mM NaCl for pH-insensitive liposomes or 1 mM HEPES (pH 7.4) for pH-sensitive liposomes, was added to the dried lipid films and the samples were incubated for 15 min at room temperature (approx. 25 °C). The hydrated lipid films were dispersed by vortex and sonication using a bath-type sonicator (AU-16C (Aiwa Co., Japan)) for 45 s. The samples were then extruded

using a Mini-extruder (Avanti Polar Lipids, Inc., USA) and were passed through filters with pore diameters of 0.1 μm and 0.2 μm (Cytiva, USA). These steps were repeated eleven times. When necessary, 50 mM calcein (Dojindo Laboratories, Japan) suspended in 1 mM HEPES buffer (pH 7.4) was added to the dried lipid films. Nonencapsulated calcein was separated using a gel-filtration column Sephadex G-50 fine **(**Cytiva**,** USA**)** packed in an Econo-Column (1.0 cm × 20 cm, Bio-Rad Laboratories, Inc., USA). The concentration of liposomes was adjusted to approx. 5.5×10^{-4} mol/L and *Rm*XeR was then added (lipid:protein=30:1 molar ratio) by the standard dilution method as described elsewhere.[3] After that, the sample was centrifuged (22,140 × *g* for 15 min) at 25 °C to remove aggregates and the supernatant was collected. The samples were stored at 4 °C and used for measurements within 1 week.

For the production of mRNA-encapsulated liposomes, we employed the ethanol dilution method as described elsewhere.[4] In short, lipids containing DOPE and CHEMS at a 7:3 molar ratio (total 4.4×10^{-7} mol) were dissolved in 108 μL 99.5% ethanol to prepare the lipid solution. The mRNA encoding EGFP (CleanCap® EGFP mRNA (5moU)) was purchased from TriLink BioTechnologies, USA. Then, 75 μL mRNA (0.44 mg/mL) was mixed with 75 μL cationic peptide protamine (0.18 mg/mL (Merck KGaA, Germany)) in a buffer containing 1 mM HEPES (pH 7.2-7.5, Thermo Fisher Scientific Life Sciences, USA) to produce mRNA-loaded nanoparticles (nitrogen/phosphate (N/P) ratio = 0.9). After that, 65 μL of the nanoparticles was mixed with 108 μL of the lipid solution, and the mixture was added to 630 μL 1 mM HEPES buffer for incorporating the nanoparticles into the liposomes. For empty liposomes, only 1 mM HEPES buffer was added instead of the nanoparticles. After diluting the resulting suspension with 4 mL 1 mM HEPES buffer, ethanol was removed by ultrafiltration using an Amicon Ultra-15-100K filter (Merck KGaA, Germany), and the retained solution was again diluted with 4 mL 1 mM HEPES buffer to replace the ethanol with 1 mM HEPES buffer. Thus, mRNA-encapsulated LiDL were constructed and *Rm*XeR was then incorporated into the liposomes by the standard dilution method as described above. After preparation of the proteoliposomes, the positively-charged peptide stearyl octaarginine (STR-R8, TORAY Industries, Inc., Japan) was added to the sample (8.5 mol% total lipid) and incubated for 30 min at room temperature (approx. 25 °C).

UV-Vis absorption spectroscopy and DLS experiments

Absorption spectra of the samples were obtained using UV-2450 and UV-2600 spectrophotometers (Shimadzu Corp., Japan) at room temperature (approx. 25 °C). Hydrodynamic diameter and polydispersity were measured by Dynamic Light Scattering (DLS) (Zetasizer μV or Zetasizer nanoZSP, Malvern Panalytical, Ltd., UK), while Zeta potentials were measured by the Zetasizer nanoZSP at 25 °C. To monitor their time-dependent changes, the liposomes were stored in the dark for 2 weeks at 4 °C after which their physicochemical properties

were measured.

Measurements of environmental pH in solution

Light-induced pH changes of the bulk solution were monitored using a pH meter equipped with a micro pH electrode (LAQUA F-72, Horiba, Ltd., Japan). The samples containing liposomes (2 mL, 1.2×10^{-4} mol/L) were kept in the dark at 25 °C for several minutes and then were illuminated with a 300 W Xe light source MAX-303 (Asahi Spectra Co., Ltd., Japan) equipped with a 550 ± 10 nm bandpass filter (Asahi Spectra Co., Ltd., Japan) for 3 min at 0.080 mW/mm^2. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Merck KGaA, Germany; final concentration of 80 μM) was used as a proton-selective ionophore.

Calcein release upon light illumination

The samples were kept in the dark and then were illuminated with a 300 W Xe light source MAX-303 equipped with a 550 ± 10 nm bandpass filter for 60 min at room temperature (approx. 25 °C). Light intensity was adjusted to 0.080 mW/mm^2 at 550 nm. Before and after light illumination, the calcein release rate was calculated as follows:

$$Calcein\ release\ (\%) = \frac{F_t - F_0}{F_{max} - F_0} \times 100$$

Where F_t is the fluorescence intensity in time, F_0 is the initial fluorescence intensity and F_{max} is the fluorescence intensity after the addition of Triton X-100 (FUJIFILM Wako Pure Chemical Industries, Ltd., Japan; final concentration of 1.0% (v/v)). Calcein fluorescence intensity was measured at 520 nm with excitation at 490 nm using a fluorescence spectrophotometer F-7100 (Hitachi High-Tech Corp., Japan).

Flow cytometry for monitoring cellular uptake

Human cervical carcinoma HeLa cells (5×10^5 cells) were cultured in 35 mm dishes (Corning, USA) with Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Gibco, DMEM/F12, Thermo Fisher Scientific Life Sciences, USA) supplemented with 10% fetal bovine serum, 0.0625% (w/v) penicillin and 0.01% (w/v) streptomycin under a 5% CO_2 atmosphere at 37 °C for 24 ± 3 hr. After the medium was replaced with serum-free Dulbecco's Modified Eagle Medium (DMEM, FUJIFILM Wako Pure Chemical Industries, Ltd., Japan), liposomes (2.75×10^{-5} mol/L) labeled with DiD (0.2 mol% to lipids (PromoCell, Germany)) were added to HeLa cells and the cells were incubated under a 5% CO_2 atmosphere at 37 °C for 1 hr. After the incubation, the cells were washed with PBS(-) once, then collected by trypsinization and resuspended in 1 mL DMEM/F12 containing 10% fetal bovine serum, 0.0625% (w/v) penicillin and 0.01% (w/v) streptomycin. After that, each cell suspension was centrifuged (500 × *g* for 5 min at 4 °C) and the

supernatant was removed. The cells were washed once by the addition of 1 mL FACS buffer (PBS(-) containing 0.5% bovine serum albumin (BSA) and 0.1% NaN_3). Finally, the cells were suspended in 0.5 mL FACS buffer. After filtering through a 42 μm nylon mesh, we evaluated the cellular uptake of liposomes by flow cytometry (Galios, Beckman Coulter Inc., USA). The cellular uptake is expressed as the mean fluorescence intensity (MFI) of DiD signals (FL6 detector; 660 nm). Several chemical reagents were used to inhibit uptake pathways; genistein (FUJIFILM Wako Pure Chemical Industries, Ltd., Japan; final concentration of 2 μg/mL) as a caveolin-dependent endocytosis inhibitor, amiloride (Cayman Chemical, USA; final concentration of 250 μM) as a micropinocytosis inhibitor and chlorpromazine (CPZ, FUJIFILM Wako Pure Chemical Industries, Ltd., Japan; final concentration of 15 μg/mL) as a clathrin-dependent endocytosis inhibitor. As a control, the cells were incubated at 4 °C to inhibit all energy dependent cellular uptake pathways. HeLa cells were preincubated with the inhibitors before the addition of liposomes under a 5% CO_2 atmosphere at 37 °C for 1 hr. Following that, inhibitors were added and the cellular uptake of liposomes was evaluated.

Intracellular trafficking observations of LiDL using CLSM

HeLa cells (8×10^4 cells) were cultured in 35 mm glass bottom dishes (Matsunami Glass Industries, Ltd., Japan) with DMEM/F12 containing 10% fetal bovine serum, 0.0625% (w/v) penicillin and 0.01% (w/v) streptomycin under a 5% CO_2 atmosphere at 37 °C for 24 ± 3 hr. After the cell medium was replaced with DMEM free of fetal bovine serum, liposomes containing calcein (2.75×10^{-5} mol/L) were added to the HeLa cells and the cells were incubated under a 5% CO_2 atmosphere at 37 °C for 1 hr. After the incubation, the dishes were washed with PBS(-) three times. The medium containing Lysotracker Deep Red (Thermo Fisher Scientific Life Sciences, USA; final concentration of 50 nM) was then added to the dishes and incubated under a 5% CO_2 atmosphere at 37 °C for 20 min. After the incubation, the medium was replaced with CO_2-independent medium (Thermo Fisher Scientific Life Sciences, USA). The cells were observed by confocal laser scanning microscopy (CLSM) (FV-1200 (Olympus Corp., Japan)) after staining the endosomes/lysosomes. The cells were excited with wavelengths of light at 473 and 635 nm for detecting calcein and DiD, respectively, in which a diode laser was used as a light source. Images were obtained using an FV-1200 equipped with a silicon objective lens (UPLSAPO 60×/NA. 1.3, Olympus Corp., Japan) and a dichroic mirror (DM405/473/559/635, Olympus Corp., Japan). Two fluorescence detection channels were set to the following filters: Channel-1: 485 nm (green) for calcein and Channel-2: 668 nm (red) for Lysotracker. To monitor light-induced cytosolic calcein release, the cells were illuminated with 559 nm light (4.3 W/mm^2) for 60 min, in which a diode laser (Olympus Corp., Japan) was used as a light source. For quantitative evaluation of colocalization between calcein and endosomes/lysosomes, we analyzed the obtained

images with Fiji software (ver. 2.9.0).[5] Then, we calculated the Mander coefficient (M) and the level before light illumination (i.e., 0 min) was normalized as 1.0.

$$Mander\ coefficient\ (M) = \frac{\sum_i G_{i,\,coloc}}{\sum_i G_i}$$

Where G_i and $Gi_{,coloc}$ represent the total pixels of calcein fluorescence signals and those of the colocalized fluorescence signals between calcein and lysotracker, respectively.

Finally, we defined the not colocalized rate between calcein and endosomes/lysosomes (i.e., fluorescence signals from lysotracker) as follows:

Not colocalized (%) = (1-Norm. M)×100.

Estimation of the entrapment efficiency of mRNA into liposomes

mRNA contents were quantified by the RiboGreen assay (Thermo Fisher Scientific Life Sciences, USA), and the entrapment efficiency of mRNA into liposomes was calculated as follows:

$$Entrapment\ efficiency\ (\%) = \frac{A - B}{B} \times 100$$

Where A and B are the fluorescence intensities of liposomes alone and with Triton X-100 (final concentration of 0.5% (v/v)), respectively. The fluorescence intensity was measured at 530 nm with excitation at 480 nm using a fluorescence spectrophotometer F-7100.

Transfection with mRNA-encapsulated LiDL

HeLa cells (10^4 cells) were cultured in 96-well plates (Thermo Fisher Scientific Life Sciences, USA) with DMEM/F12 containing 10% fetal bovine serum, 0.0625% (w/v) penicillin and 0.01% (w/v) streptomycin under a 5% CO_2 atmosphere at 37 °C for 24 ± 3 hr. After the cell medium was replaced with DMEM free of fetal bovine serum, liposomes were added to the HeLa cells with 20 ng mRNA labeled with DiD (0.2 mol% to lipids) and the cells were incubated under a 5% CO_2 atmosphere at 37 °C for 1 hr. After the incubation, cells were washed with PBS(-). Then, DMEM/F12 containing 10% fetal bovine serum, 0.0625% (w/v) penicillin and 0.01% (w/v) streptomycin was added and incubated under a 5% CO_2 atmosphere at 37 °C. The cells were continuously illuminated at 545 ± 10 nm using a fluorescence mirror unit (U-MRFPHQ (Olympus Corp., Japan)) with an LED light source (U-HGLGPS (Olympus Corp., Japan)) for 1 hr, then incubated for 23 hr (a total of 24 hr) under a 5% CO_2 atmosphere at 37 °C. The intensity of the excitation light was adjusted to 0.023 mW/mm^2. Bright field and EGFP fluorescence images were

obtained using a fluorescence microscope (IX71 (Olympus Corp., Japan)) equipped with a CCD camera (ORCA-AG (Hamamatsu Photonics, Japan)), a fluorescence mirror unit (U-MGFPHQ (Olympus Corp., Japan)) and a LED light source (U-HGLGPS) as an excitation source. Exposure time was adjusted to 500 ms for the detection of EGFP fluorescence signals. The obtained images were analyzed with Fiji software (ver. 2.9.0).[5] The results of three or four independent experiments were averaged. EGFP-positive HeLa cells were defined as cells showing a larger green fluorescence signal than the pseudo signal from untransfected cells.

2. Schematics of pH-sensitive liposomes and the estimated physicochemical parameters of proteoliposomes

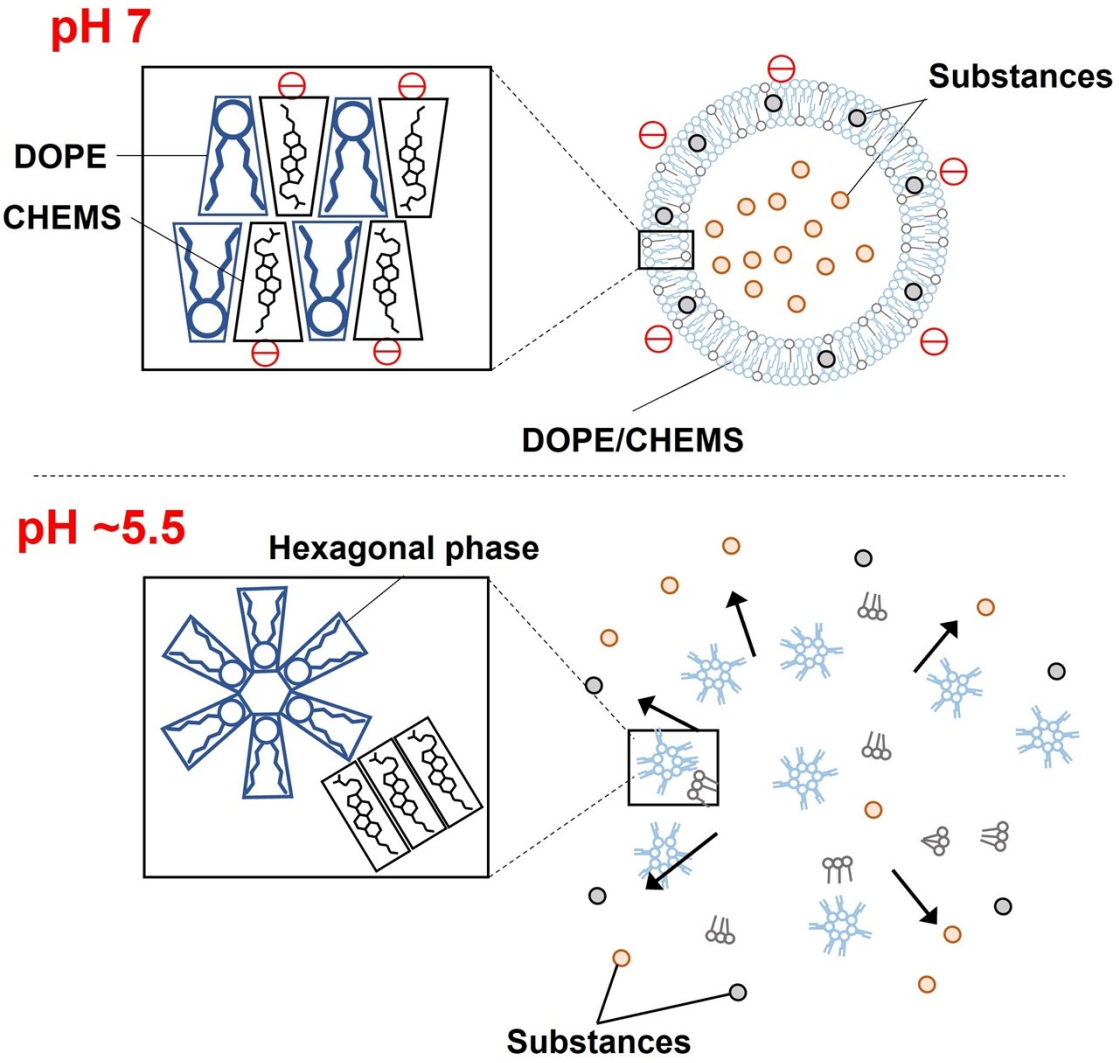

Figure S1. Schematic of pH-sensitive liposomes consisting of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS) at a 7:3 molar ratio. They are stable at a physiological neutral pH around 7.0 and are expected to be disrupted upon acidification under pH 5.5 due to the phase separation of the DOPE/CHEMS lipid bilayer.

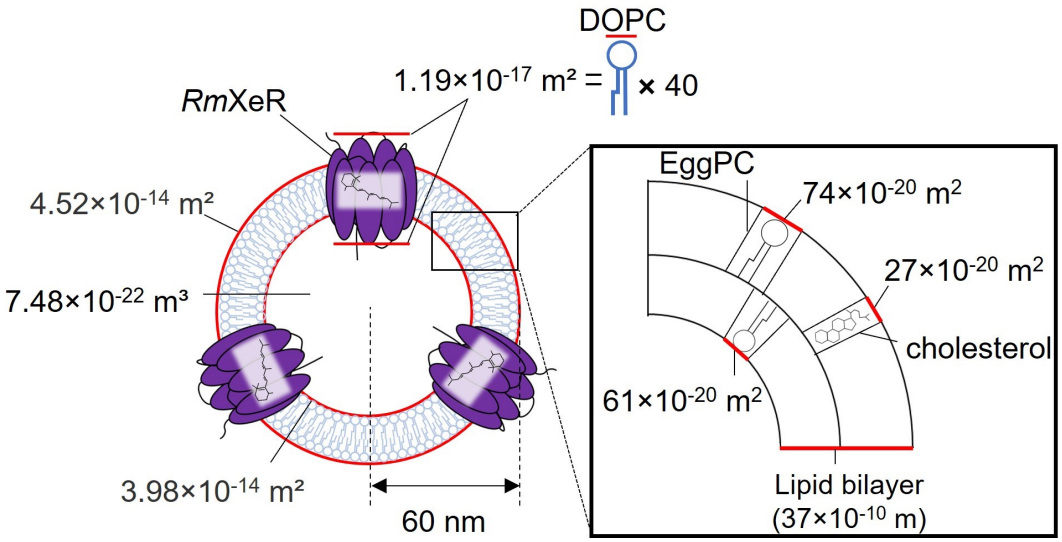

Figure S2. Estimated parameters of the lipid bilayer vesicles containing *Rm*XeR**.** Several parameters were estimated as follows; size of the proteoliposome (120 nm), surface area of the lipid bilayer (outer lipid bilayer; 74×10^{-20} m^2 and inner lipid bilayer; 61×10^{-20} m^2 for EggPC), thickness (37×10^{-10} m for EggPC)[6], surface area of cholesterol in the hydrated dipalmitoyl phosphatidylcholine (DPPC) bilayers containing 25-40% cholesterol (27×10^{-20} m^2)[7], and the size of the bacteriorhodopsin monomer (corresponding to 40 lipid molecules).[8]

3. CLSM images of HeLa cells

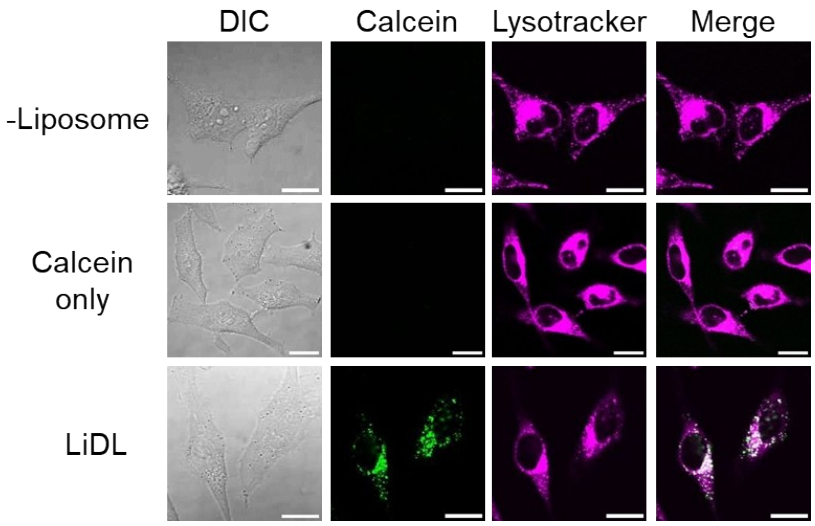

Figure S3. CLSM images of HeLa cells after the uptake of calcein-encapsulated LiDL. Images show DIC (Differential Interference Contrast) and fluorescence images. Green, magenta and white signals represent calcein fluorescence, endosome or lysosome fluorescence, and their colocalization, respectively. As a negative control, we obtained images of HeLa cells without LiDL and with calcein alone (final concentration of 2 μM). Scale bars indicate 20 μm.

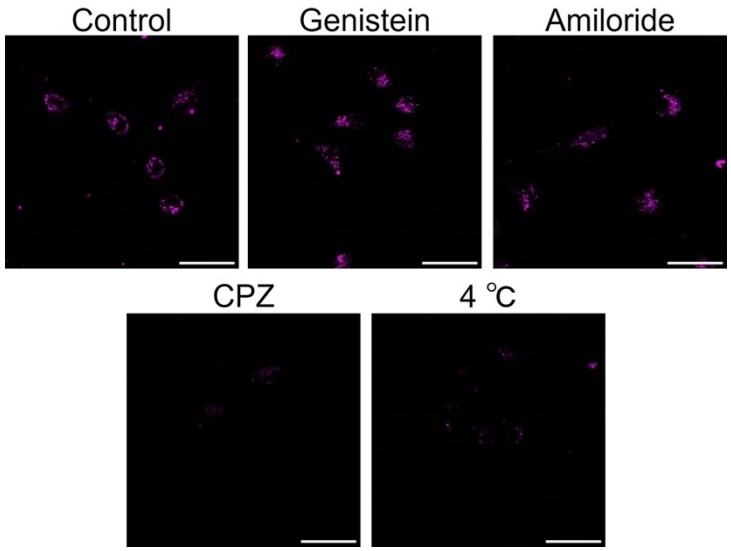

Figure S4. CLSM images of HeLa cells after the uptake of LiDL with chemical endocytosis inhibitors and at 4 ˚C. The fluorescence signals of DiD are shown as magenta. Scale bars indicate 50 μm.

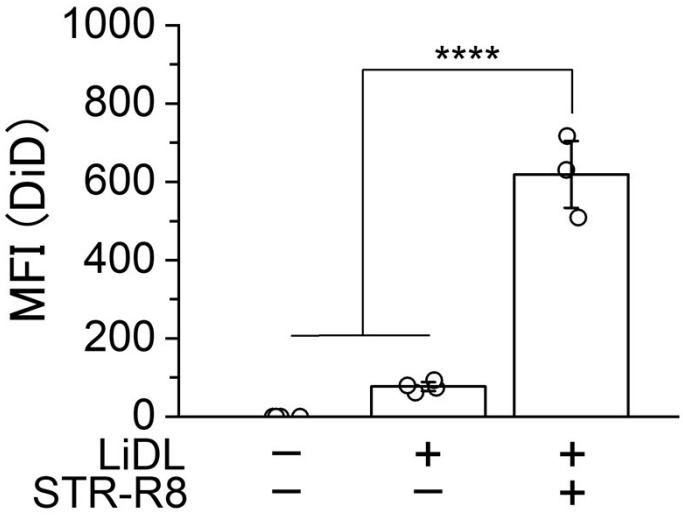

Figure S5. Flow cytometry experiment to evaluate the cellular uptake of mRNA-encapsulated LiDL modified by STR-R8 (mRNA LiDL-R8). LiDL were labeled with a lipophilic carbocyanine dye DID which shows red fluorescence. The cellular uptake is expressed as the mean fluorescence intensity (MFI) of DiD signals. Error bars represent the standard deviation (n=3-5). Asterisks indicate significant differences from the value of mRNA LiDL-R8 (****$p < 0.0001$; Dunnett's test).

4. List of physicochemical properties

Table S1. Physicochemical properties of proteoliposomes (Size, PdI and ζ-potential) (n=3-4, mean ± standard deviation)

Sample	Size (nm)	PdI	ζ-potential (mV)
EggPC/cholesterol (7:3) liposomes with *Rm*XeR	119 ± 7.29	0.20 ± 0.019	ND
DOPE/CHEMS (7:3) liposomes alone (- *Rm*XeR)	176 ± 4.68	0.12 ± 0.021	ND
DOPE/CHEMS (7:3) liposomes with *Rm*XeR (LiDL)	170 ± 4.16	0.15 ± 0.019	-42 ± 3.4

*ND; not determined

Table S2. Physicochemical properties of mRNA-encapsulated liposomes (Size, PdI and ζ-potential) (n=3-4, mean ± standard deviation)

Sample	Size (nm)	PdI	ζ-potential (mV)
DOPE/CHEMS (7:3) liposomes alone (-*Rm*XeR)	268 ± 39.1	0.33 ± 0.072	-43 ± 8.1
DOPE/CHEMS (7:3) liposomes with *Rm*XeR (LiDL)	210 ± 27.1	0.24 ± 0.091	-46 ± 12
DOPE/CHEMS (7:3) liposomes with *Rm*XeR and STR-R8 (LiDL-R8)	218 ± 23.4	0.24 ± 0.074	29 ± 5.8

5. References

1. S. Inoue, S. Yoshizawa, Y. Nakajima, K. Kojima, T. Tsukamoto, T. Kikukawa and Y. Sudo, *Phys. Chem. Chem. Phys.*, 2018, **20**, 3172-3183.
2. Y. Yamada, H. Akita, H. Kamiya, K. Kogure, T. Yamamoto, Y. Shinohara, K. Yamashita, H. Kobayashi, H. Kikuchi and H. Harashima, *Biochim. Biophys. Acta, Biomembr.*, 2008, **1778**, 423-432.
3. J.-L. Rigaud and D. Lévy, *Methods Enzymol.,* 2003, **372**, 65-86.
4. Y. Yamada, K. Somiya, A. Miyauchi, H. Osaka and H. Harashima, *Sci. Rep.*, 2020, **10**, 1-13.
5. J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak and A. Cardona, *Nat. Methods*, 2012, **9**, 676-682.
6. C. Huang and J. Mason, *Proc. Natl. Acad. Sci. U.S.A.*, 1978, **75**, 308-310.
7. C. Hofsäß, E. Lindahl and O. Edholm, *Biophys. J.*, 2003, **84**, 2192-2206.
8. M. Orwick-Rydmark, J. E. Lovett, A. Graziadei, L. Lindholm, M. R. Hicks and A. Watts, *Nano Lett.*, 2012, **12**, 4687-4692.