Electronic Supplementary Information (ESI) for

Size-Dependent Uptake of Electrically Neutral Amphipathic Polymeric Nanoparticles by Cell-sized Liposomes and an Insight into Their Internalization Mechanism in Living Cells

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Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, USA). Chloroform, diethylether, acetone, *N,N*′-methylenebisacrylamide (MBAA), 2,2'-azobis(2-methylpropionamidine)dihydrochloride (V-50), NaOH, HCl, 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), and potassium Eu-encapsulated Preyssler-type phosphotungstate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium sulfate, *N*-isopropylacrylamide (NIPAm), hexane, methanol, ganglioside GM1, Accutase™, Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose), Dulbecco's phosphate-buffered saline without Ca and Mg (D-PBS), and penicillin-streptomycin mixed solution were purchased from Nacalai Tesque (Kyoto, Japan). Acryloyl chloride, 4-amino fluorescein (λ_{ex} = 490 nm, λ_{em} = 520 nm), and Alexa Fluor® 594-conjugated cholera toxin subunit B (CTB-594, λ_{ex} = 590 nm, λ_{em} = 617 nm) were purchased from Tokyo Chemical Industry (Tokyo, Japan). *N*-(Rhodamine red-X)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (Rho-DHPE, λ_{ex} = 560 nm, λ_{em} = 580 nm) and LysoTracker® Deep Red were purchased from Thermo Fisher Scientific (Waltham, USA). Deionized water was obtained from a Millipore Milli-Q purification system. 2-Methacryloyloxyethyl phosphorylcholine (MPC) was purchased from NOF Corporation (Tokyo, Japan). Calcein and calcein-AM were obtained from Dojindo Laboratories (Kumamoto, Japan). Fluorescein acrylamide (FAm) was synthesized by reacting 4-amino fluorescein and acryloyl chloride as described in the previous paper [1].

Preparation of fluorescein-conjugated amphipathic nanoparticles (FL-NPs) (Fig. S1-S3)

FL-NPs were prepared by emulsifier-free precipitation polymerization of NIPAm (407 mg, 3.6 mmol), MBAA (30.8 mg, 0.2 mmol), MPC (59. mg, 0.2 mmol), and FAm (4.0 mg, 0.01 mmol) with V-50 (54.3 mg, 0.2 mmol) as an initiator in 10 mM Tris-HCl buffer (pH 7.4) at 70° C for 12 h. The obtained particles were purified by size exclusion chromatography using Sephadex G-100 with 10 mM Tris-HCl buffer (pH 7.4) as the eluent. The volume-based average particle size of the obtained FL-NPs was ca. 8.4 nm measured by DLS at 25°C. The maximum absorption and emission wavelengths of the FL-NPs were 504 and 526 nm, respectively, measured using a V-560 spectrophotometer (JASCO Ltd., Tokyo, Japan) and a fluorescence spectrophotometer (F-2500, HITACHI, Tokyo, Japan). For examining the amphipathicity of FLNPs, an octanol-water partitioning coefficient was estimated spectrophotometrically in a mixture of 1-octanol (3 mL) and water (3 mL) containing FL-NPs (final concentration: 1.05 mg/mL) using V-560. Particle size, size distribution, and ζ-potential were measured using a dynamic light scattering system (DLS, Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, UK). For TEM sample preparation, FL-NPs were purified by size

exclusion chromatography using Sephadex G-100 with pure water, and the dispersion was dried on carbon-coated Cu-grid. The FL-NPs-dried Cu-grid inverted on 1 wt% phosphotungstate aqueous solution, then the sample was dried before TEM observation (JEM-1230, JEOL.Ltd).

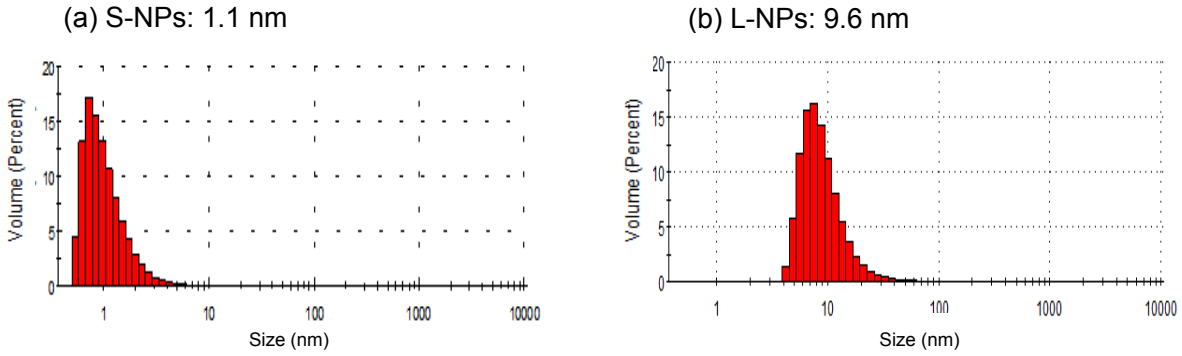

Figure S1. Particle size distribution and volume-based average particle sizes of S-NPs (a) and L-NPs (b) measured by DLS. The ζ-potentials of S-NPs and L-NPs are +2.9 mV and -1.1 mV, respectively.

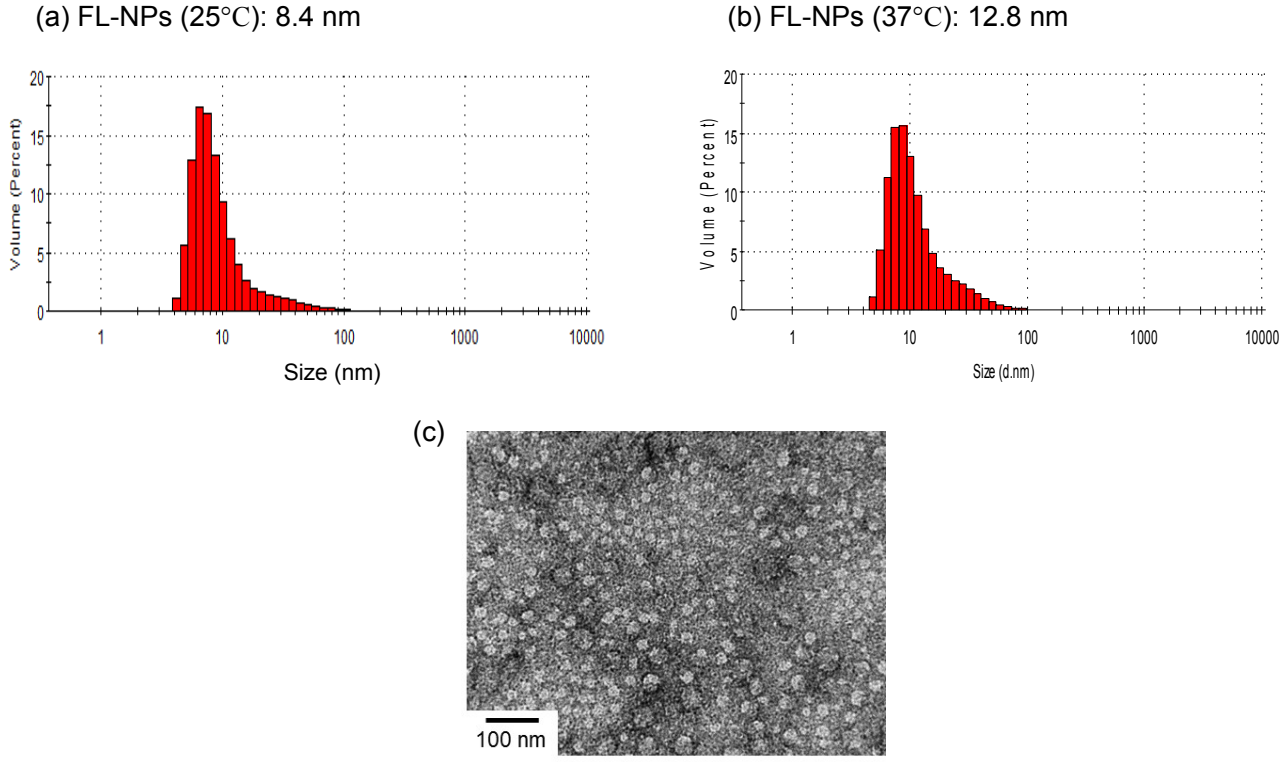

Figure S2. Particle size distribution and volume-based average particle size of FL-NPs measured by DLS (a: 25°C; b: 37°C) and transmission electron microscope (TEM) image of FL-NPs (c). The ζ-potentials of FL-NPs is +1.58 mV.

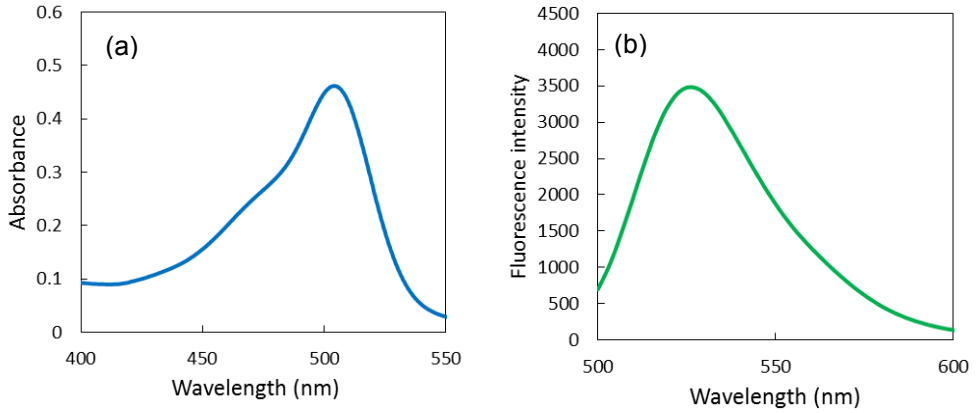

Figure S3. UV-Vis (a) and fluorescence (b) spectra for FL-NPs prepared by the emulsifier-free emulsion polymerization measured at 25 °C. Excitation wavelength for fluorescent measurement was 505 nm.

The obtained FL-NPs were fractionated into a smaller portion and a larger portion using an ultrafiltration membrane with a fractionation molecular weight of 10 kDa (Amicon Ultra, 4 mL, Merck Millipore, 4,000 × *g*, 20 min). The FL-NPs containing the filtrate and the residue were designated as S-NPs and L-NPs, respectively. The S-NPs were further purified using an ultrafiltration membrane with a fractionation molecular weight of 3 kDa (Amicon Ultra, 15 mL, Merck Millipore, 14,000 × *g*, 20 min). The volume-based average particle sizes of the obtained S-NPs and L-NPs were ca. 1.1 nm and 9.6 nm, respectively, measured by DLS at 25°C.

Preparation of cell-sized liposomes (Fig. S4 and S5)

Cell-sized liposomes were prepared by the gentle hydration method [2-4]. Lipids and Rho-DHPE were dissolved in chloroform: methanol (2:1 v/v) to prepare 2.0 mM and 0.10 mM solutions, respectively. The lipid solution (20 μL) and Rho-DHPE (2 μL) were transferred to a glass tube, and the organic solvent was evaporated using nitrogen gas, leading to a thin lipid film. The thin film was subsequently dried under vacuum for 3 h, and hydrated with 10 mM Tris-HCl buffer (pH 7.4, 200 μL) at 55°C overnight. The final lipid concentration was 0.20 mM. The prepared compositions for homogeneous and heterogeneous liposomes were in molar ratios of DOPC: DPPC: cholesterol = 100:0:0 (DOPC liposomes), 0:67:33 DPPC-chol liposomes), 40:40:20 (DOPC-DPPC-chol liposomes), and 50:50:0 (DOPC-DPPC liposomes).

(a)

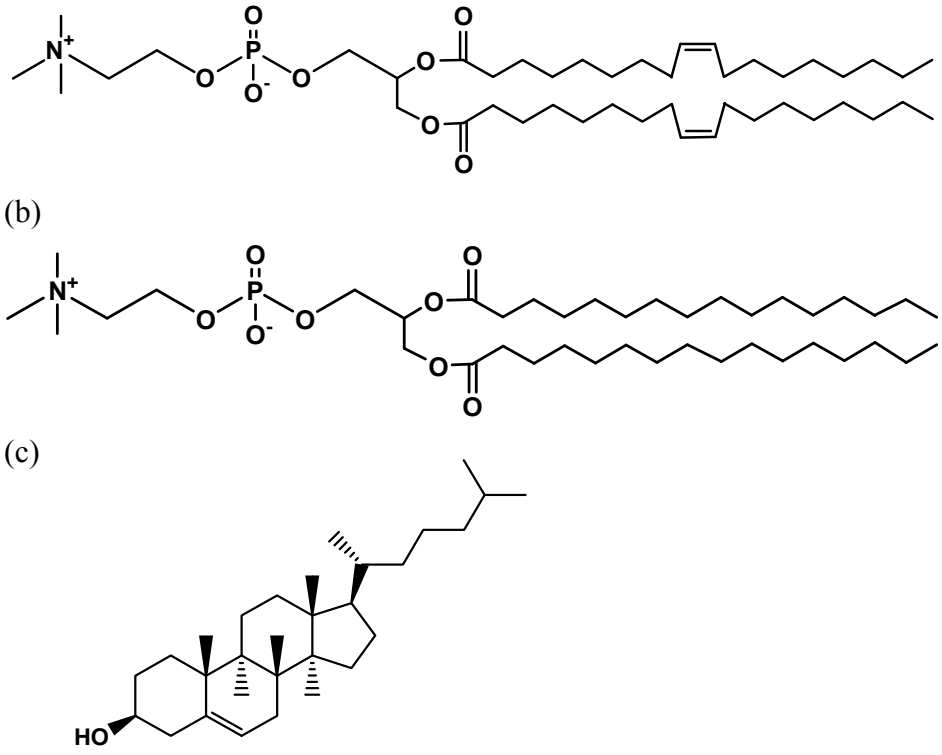

Figure S4. Chemical structures of (a) 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), (b) 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and (c) cholesterol.

Liposomes with stained phase-separated regions were also prepared using the same procedure. Lipid films were prepared with the molar ratio of DOPC: DPPC: cholesterol = 40:40:20 containing 1 mol % GM1. The films were hydrated with 10 mM Tris-HCl buffer (100 μL) at 37°C for 1 h. Then, the solution was mixed with 10 mM Tris-HCl buffer (pH 7.4, 100 μL) containing 50 μg mL^{-1} CTB-594 [3].

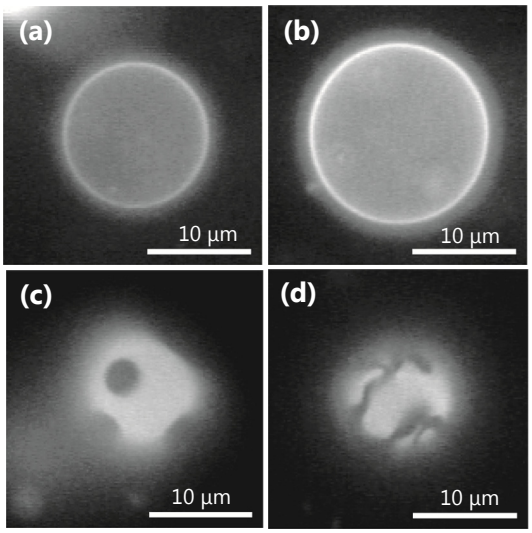

Figure S5. Cell-sized liposomes prepared by gentle hydration method in 10 mM Tris-HCl buffer (pH 7.4). (a) DOPC liposomes (14.0 μm), (b) DPPC-chol liposomes (17.2 μm), (c) DOPC-DPPC-chol liposomes (10.7 μm), and (d) DOPC-DPPC liposomes (11.9 μm) were prepared using various lipid compositions (molar ratios of DOPC: DPPC: chol = 100:0:0, 0:67:33, 40:40:20, and 50:50:0, respectively). Bright regions in (c) and (d): Rho-DHPE-localized DOPC-rich solid-disordered (L_d) phase; dark region in (c): liquid-ordered (L_o) phase; dark region in (d): solid-ordered (S_o) phase.

Determination of logP for FL-NPs (Fig. S6)

FL-NPs were purified by size exclusion chromatography using Sephadex G-100 with pure water as an eluent. The dispersion (2 mL, solid content: 2.1 mg/mL) was dried in vacuo. The dried FL-NPs were re-dispersed with water-saturated 1-octanol (2 mL). The FL-NPs dispersion in 1-octanol was diluted with aqueous-saturated 1-octanol to draw a calibration curve of FL-NPs using UV-Vis spectroscopy (λ = 508 nm).

For determination of logP of FL-NPs, FL-NPs dispersed in pure water (3 mL, solid content: 2.1 mg/mL) was incubated with 1-octanol (3 mL) at 37° C for 24 h. During the incubation, the volume change of both phases was negligible. The absorbance at 508 nm in the 1-octanol phase was measured to determine the FL-NPs concentration in the 1-octanol phase ($FL\text{-}NPs_{org}$) using the prepared calibration curve. The FL-NPs

concentration in the aqueous phase (FL-NPs$_{aq}$) can be calculated by subtracting the FL-NPs concentration in 1-octanol phase from the initial concentration. A Log P values was calculated to be -0.08 by *Eq*. S1

$$\text{Log P} = \log(\text{FL-NPs}_{org}/\ \text{FL-NPs}_{aq}) \qquad \text{(S1)}$$

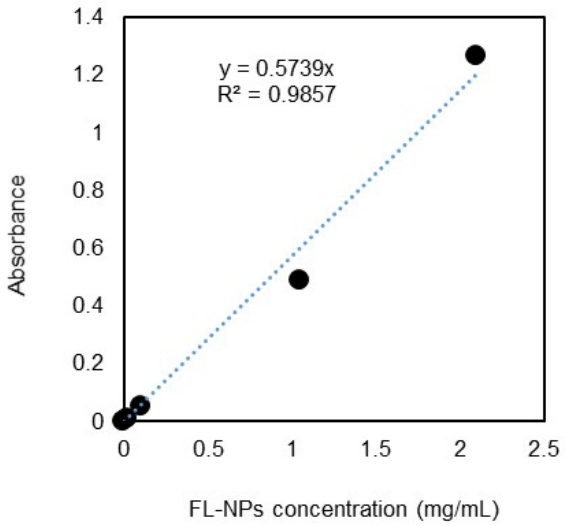

Figure S6. Calibration curve of FL-NPs concentration in 1-octanol (λ =508 nm).

CLSM observation and uptake rate analysis (Fig. S7)

FL-NPs (0.54% wt.) and liposome dispersions (0.20 mM) were mixed (1:1 v/v) in a microtube. The mixed solution was immediately placed in a silicon well (thickness of 200 μm) on a glass slide and covered with a cover glass. Observations were performed for a total period of 3 h using CLSM (IX81, Olympus, Tokyo, Japan) at 25°C using filters U-MWIG3 (λ_{ex} = 550 nm, λ_{em} = 580 nm) for rhodamine, U-MWIY2 (λ_{ex} = 590 nm, λ_{em} = 617 nm) for Alexa Fluor® 594, and U-MWIB3 (λ_{ex} = 495 nm, λ_{em} = 519 nm, Olympus) for fluorescein. The objective lens was UPlanSApo (Olympus, 60×, N.A. = 1.35), and the multi Ar laser had a wavelength conversion Mid-IR Laser Opti λ 3360 (NTT Electronics, Kanagawa, Japan) and GLS3135 (Showa Optronics, Tokyo, Japan). Fluorescence microscopic images were also obtained using a fluorescence microscope IX73 (Olympus) equipped with U-FGW (Olympus) as the filter.

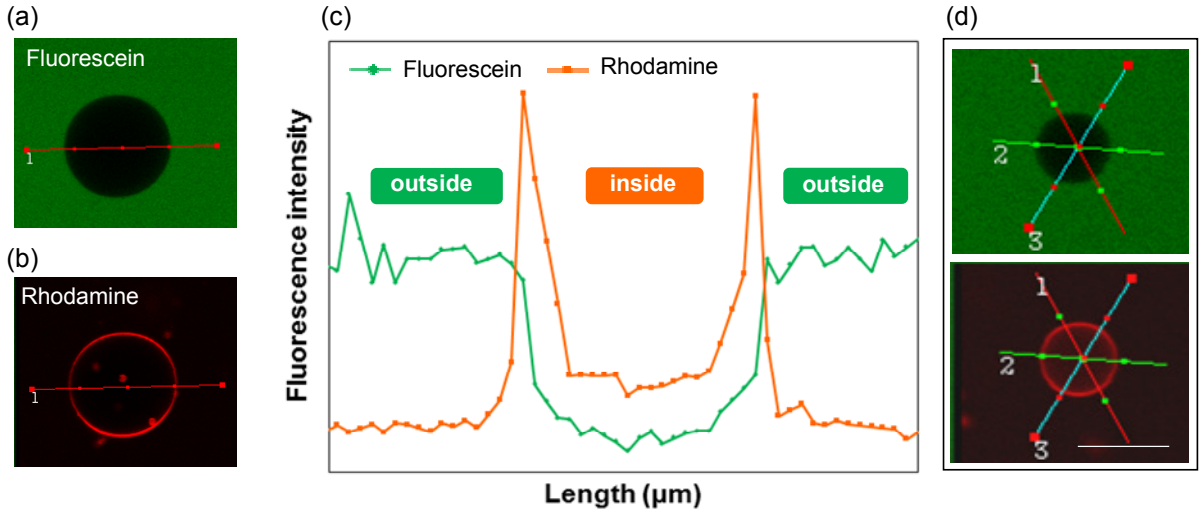

Figure S7. A method for measuring the fluorescence intensity of FL-NPs inside and outside cell-sized liposomes by confocal laser scanning microscopy (CLSM). (a) CLSM images of FL-NPs (fluorescein); (b) CLSM images of Rho-DHPE in the liposome membrane; (c) fluorescent intensities calculated by the one-dimensional analysis of the CLSM images (see red lines in a and b); (d) fluorescence intensity was measured in triplicate at different points of the same liposomes. Scale bar is 10 μm.

To estimate the apparent uptake rate of FL-NPs in the liposomes after the administration of FL-NPs, the CLSM images were analyzed using the following method. Fluorescence intensities derived from Rho-DHPE and FL-NPs were quantified one-dimensionally by CLSM, where two peak maxima derived from Rho-DHPE were defined as the boundary of the liposome membrane.

The average fluorescence intensities on the inside and outside of the liposomes at time t (min) are defined as $I_{i,t}$ and $I_{o,t}$, respectively. The net intensity is calculated by *Eq.* S2 to correct blurring of the laser source and quenching during the measurements, where $I_{o,0}$ is the initial intensity (0 min).

$$I_{i,t} = apparent\ I_{i,t} \times \frac{I_{o,0}}{I_{o,t}} \qquad \text{(S2)}$$

A calibration curve for FL-NP concentration was plotted by measuring the fluorescence intensities at various concentrations of FL-NP dispersions. FL-NP concentrations on the inside of the liposomes ($C_{i,t}$) were calculated from the linear calibration curve shown in *Eq.* S3 and the obtained $I_{i,t}$ values. The change in FL-NP concentration on the inside of the liposomes, $\Delta C_{i,t}$, was evaluated from *Eq.* S4, where $C_{i,0}$ is the initial concentration of the FL-NPs on the outside (0 min).

$$C_{i,t} = 3957.9 \times I_{i,t} + 15.646 \quad \text{(S3)}$$

$$\Delta C_{i,t} = C_{i,t} - C_{i,0} \quad \text{(S4)}$$

The uptake rates of FL-NPs were measured three times with three different cell-sized liposomes for DOPC and DOPC-DPPC-chol liposomes. The significant difference was observed between these liposomes, i.e. the student's t test p value was calculated to be less than 0.05 after 3.5×10^3 sec. The uptake rate constant (k) is calculated from measuring $C_{i,t}$ and $C_{o,0}$ in *Eq.* S5. *Eq.* S6 was generated by integrating *Eq.* S5, assuming that $C_{o,0}$ is certain since the concentration of FL-NPs on the inside of the liposomes is much smaller than that of bulk FL-NPs, when the boundary conditions of t and C_t are from 0 to t and 0 to $C_{i,t}$, respectively. Finally, the uptake rate constant of FL-NPs was calculated by fitting in *Eq.* S6.

$$\frac{dC_i}{dt} = k(C_{o,0} - C_{i,t}) \quad \text{(S5)}$$

$$C_{i,t} = C_{o,0}\{1 - \exp(-kt)\} \quad \text{(S6)}$$

$C_{o,0}$ was calculated using Eq. S7, S8, and S9. The weight of FL-NPs added was calculated using the solid content (0.54% wt.) and the weight of the FL-NP dispersion used (x g). The volume of medium (water) was calculated using *Eq.* S8, where 1.00 g/mL was used as the density of water. When the liposome dispersion was mixed with the FL-NP dispersion at a one-to-one ratio, $C_{o,0}$ was given by *Eq.* S9.

$$\text{Weight of FL-NPs used (mg)} = x \times \frac{0.54}{100} \quad \text{(S7)}$$

$$\text{Volume of medium (mL)} = x \times \frac{100-0.54}{100 \times 1.00} \quad \text{(S8)}$$

$$C_{o,0} = \frac{0.0054x}{0.9946x \times 2} = 2.71 \text{ mg/mL} \quad \text{(S9)}$$

Size-dependent uptake of S-NPs and L-NPs by NIH3T3 cells

The murine cell line NIH3T3 known known as a commonly used standard fibroblast cell line was cultured to subconfluency in DMEM. Adherent cells were lysed using Accutase™ and seeded in dishes (1.5×10^5 cells/dish). After 24 h of incubation in a CO_2 incubator (37°C, 5% CO_2), S-NPs or L-NPs (final concentration: 5.47 μg/mL) were administered to the dishes and incubated for 24 h (37°C, 5% CO_2). LysoTracker® Deep Red (final concentration: 75 nM) was added and incubated for 1 h (37°C, 5% CO_2). After washing with fresh DMEM, CLSM observation was conducted under the same conditions as those for cell-sized liposomes. Almost no particle size change was observed at 37°C, compared with the size at 25°C (Figure S2-b), which is consistent with our previous results, [1] where the LCST of PNIPAm-based nanogels was found to be elevated to approximately 60°C, when a hydrophilic monomer MPC was copolymerized.

Temperature-dependent uptake of calcein-AM, calcein, S-NPs, and L-NPs in NIH3T3 cells

The temperature-dependent uptake of calcein, calcein-AM, S-NPs, and L-NPs in NIH3T3 cells was examined using a FACSCalibur HG flow cytometer with a filter for FITC (λ_{ex} = 530 nm) (Becton, Dickinson and Company, Franklin Lakes, USA). The murine cell line NIH3T3 was cultured to subconfluency in DMEM. Adherent cells were lysed using Accutase™ and seeded in dishes (7.0×10^5 cells/dish). After 24 h of incubation in a CO_2 incubator (37°C, 5% CO_2), calcein was administrated (final concentration: 100 μg/mL) and incubated for 3 h in a CO_2 incubator (4°C or 37°C, 5% CO_2). For the uptake at 4°C, the cells were pre-incubated for 30 min at 4°C before calcein administration. For calcein-AM, the final concentration was 5 μg/mL with 45 min incubation. S-NPs and L-NPs were incubated for 24 h at the final concentration of 5.47 μg/mL. After washing with D-PBS, adherent cells were lysed using Accutase™, and the cells were washed with D-PBS. The cells were then re-dispersed in 1 mL D-PBS and applied to the flow cytometer.

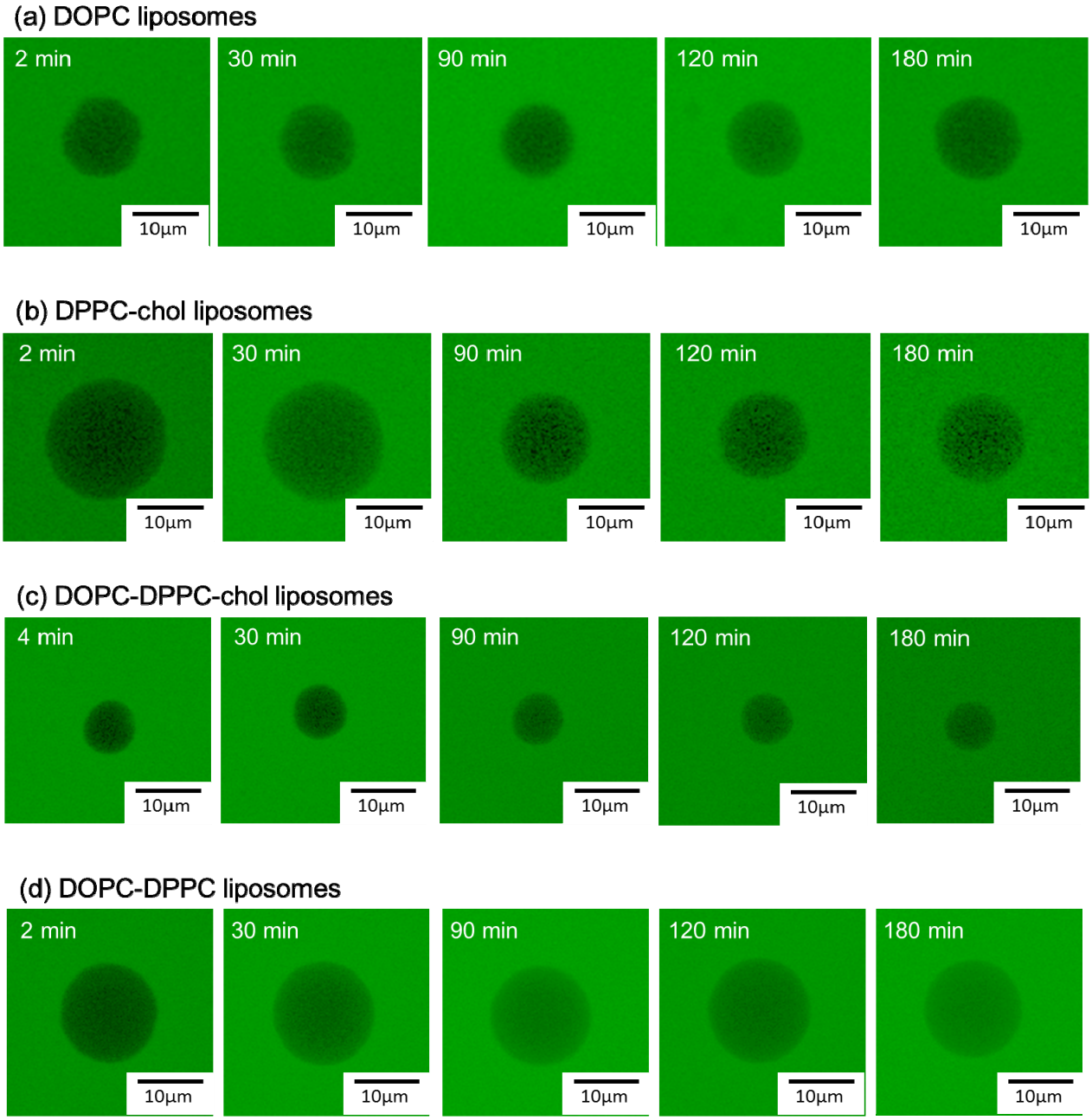

Figure S8. Confocal laser scanning microscopy (CLSM) images of FL-NPs (Fluorescein) at various times in (a) DOPC liposomes, (b) DPPC-chol liposomes, (c) DOPC-DPPC-chol liposomes, and (d) DOPC-DPPC liposomes. The slight size change of liposome in the CLSM image was caused by the Z position deviation of the cross section in the cell-sized liposomes.

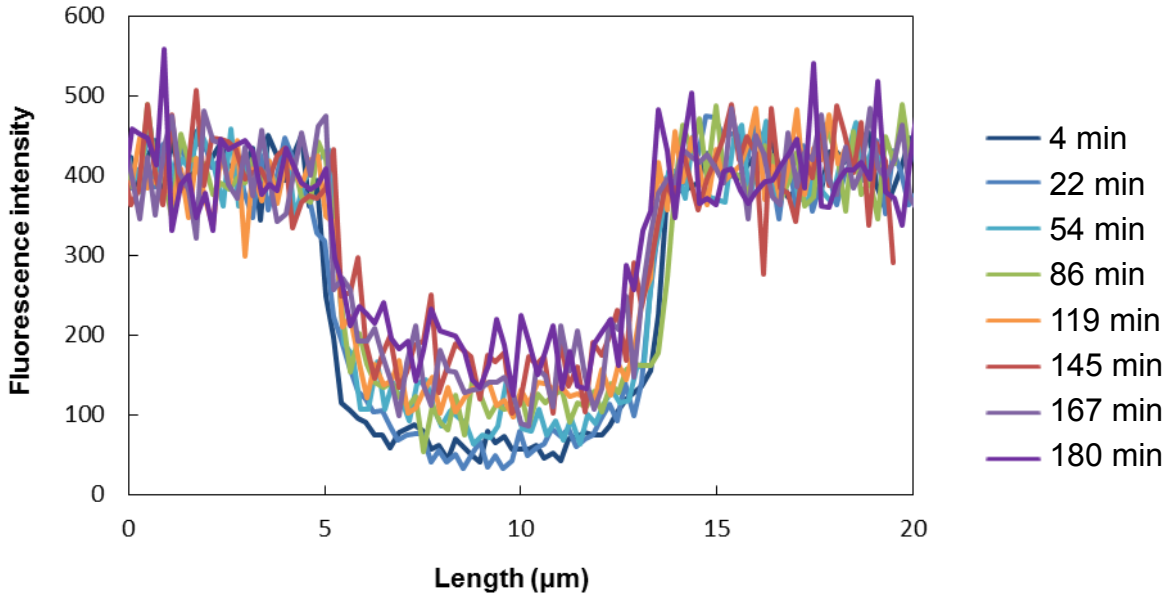

Figure S9. Raw data of CLSM for the time-dependent fluorescent intensity change in a homogeneous DOPC liposome by the FL-NP uptake.

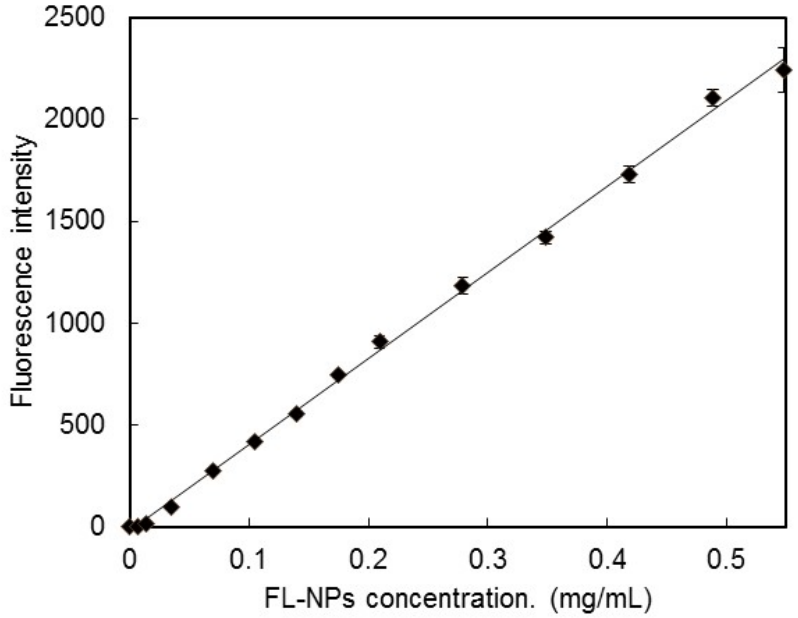

Figure S10. Calibration curve prepared by CLSM for determining FL-NPs concertation in 10 mM Tris-HCl buffer (pH 7.4). n=3.

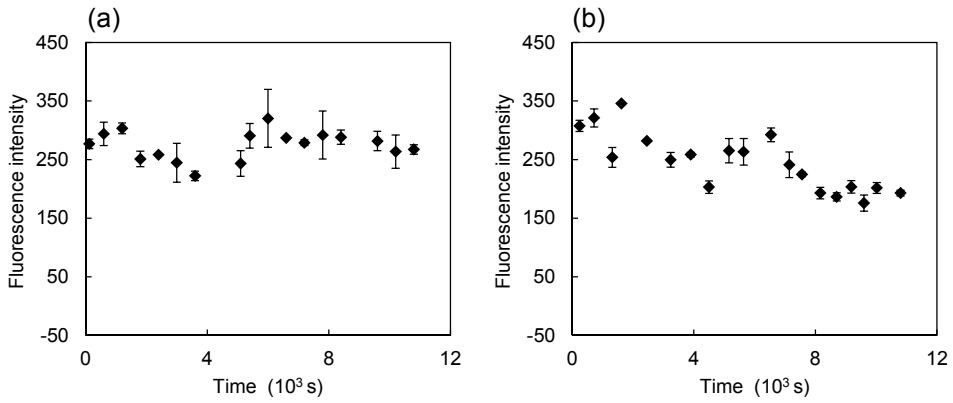

Figure S11. Fluorescence intensities derived from (a) Rho-DHPE-labeled L_d and (b) GM1/CTB-594-labeled L_o phases inside DOPC-DPPC-chol liposomes at various incubation times.

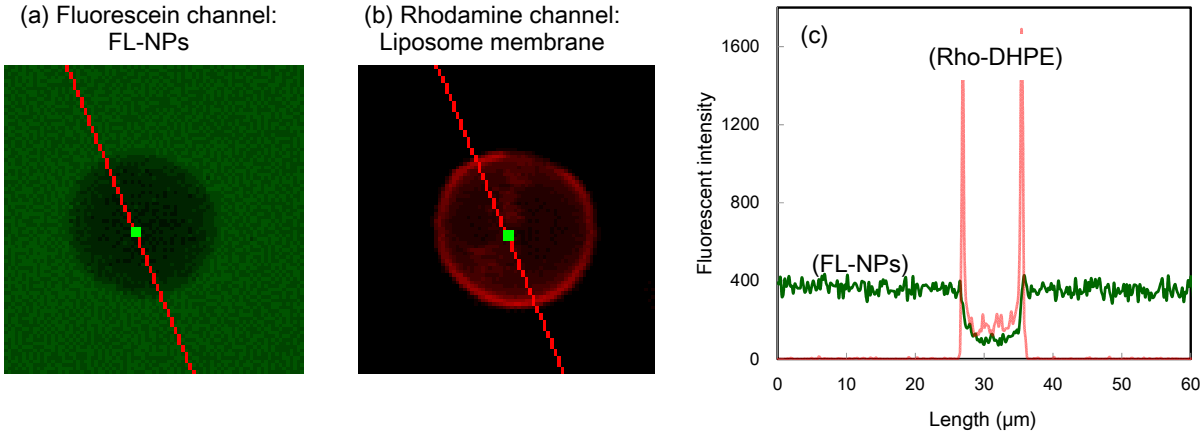

Figure S12. Confocal laser scanning microscopy (CLSM) images of FL-NPs (Fluorescein) (a) and Rho-DHPE in the DOPC/DPPC/chol liposome membrane (b), and fluorescent intensities calculated by the one-dimensional analysis (see red lines in a and b) of the CLSM images (c).

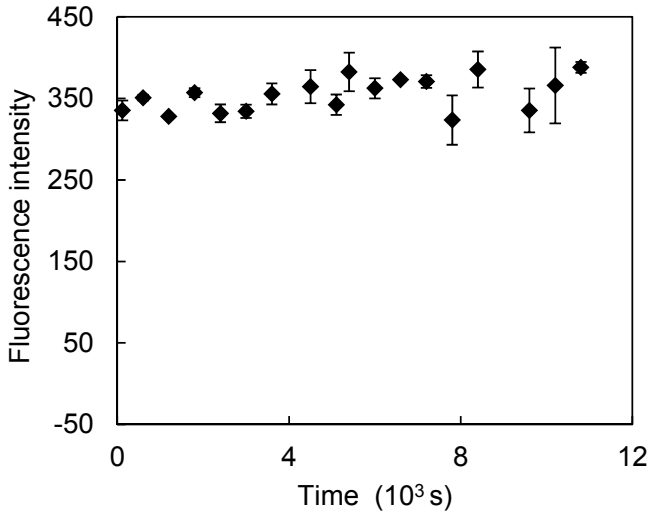

Figure S13. Fluorescence intensities derived from FL-NPs on the lipid membrane (a) and the raw data of time-dependent fluorescent intensity change inside DOPC/DPPC/chol liposome s by the FL-NP uptake measured by confocal laser scanning microscopy.

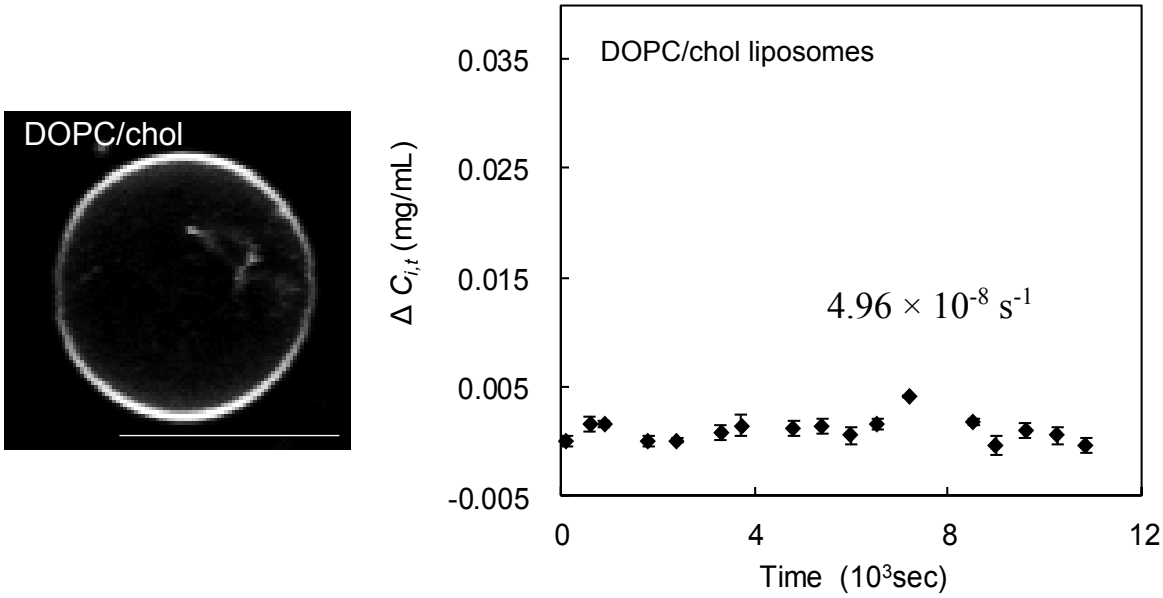

Figure S14. Fluorescent microscope image (left) and time course of the concentration change of FL-NPs on the inside of the DOPC-chol liposome. The concentration change of FL-NPs on the inside of the liposomes, $\Delta C_{i,t}$, is calculated using *Eq.* S4.

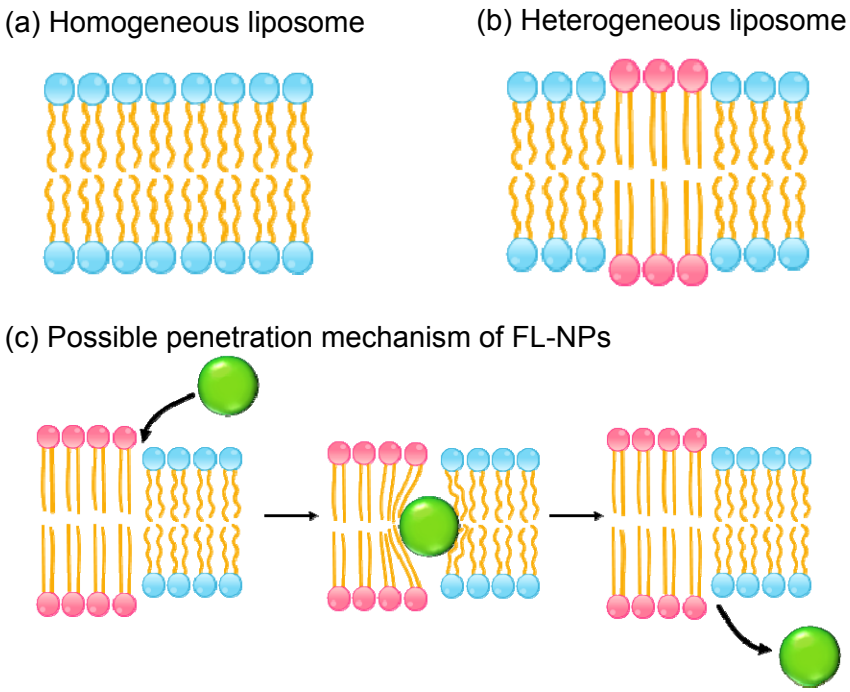

Figure S15. Schematic illustrations of membranes of (a) homogeneous liposomes, (b) heterogeneous liposomes, and (c) a possible mechanism of penetration into the liposomes.

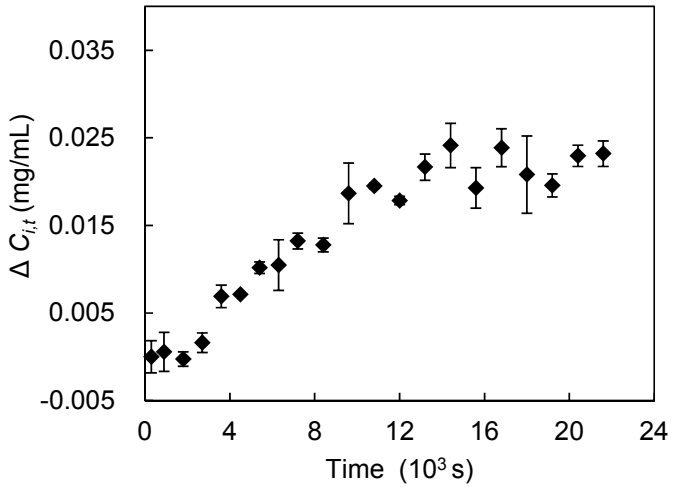

Figure S16. Relative FL-NP concentrations on the inside of the liposomes after 6 h of incubation with DOPC-DPPC-chol. $\Delta C_{i,t}$ is calculated by *Eq.* S4.

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