Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Delivery of external proteins into the cytoplasm using protein capsules modified with IgG on the surface, created from the amphiphilic two helix-bundle protein OLE-ZIP

Kousuke Takahashi,[a] Taiki Nishiyama,[a] Naoki Umezawa,[b] Yasumichi Inoue,[b] Isamu Akiba,[c] Takehisa Dewa,[a,d] Atsushi Ikeda[e] and Toshihisa Mizuno[a, d]*

[a]*Department of Life Science and Applied Chemistry, Graduate School of Engineering, Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya, Aichi 466-8555, Japan.*
[b]*Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan.*
[c]*Applied Chemistry Program, Graduate School of Advanced Science and Engineering, Hiroshima University, 1-4-1 Kagamiyama, Higashi-Hiroshima 739-8527, Japan.*
[d]*Department of Nanopharmaceutical Sciences, Graduate School of Engineering, Nagoya Institute of Technology, Gokiso-cho Showa-ku, Nagoya, Aichi 466-8555, Japan*
[e]*Faculty of Environmental Engineering, the University of Kitakyushu, 1-1 Hibikino, Wakamatsu, Kitakyushu, Fukuoka 808-0135, Japan.*

Trx-OLE-ZIP

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDE
Trx A
IADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAAT

KVGALSKGQLKEFLDANLAGSGSGHMHHHHHHSSGLVPRGSAMAA
NZ
LKKELQANKKELAQLKWELQALKKELAQGGSGGSFGLTGITLVGT
OLE(56-106)
VIGLALATPLFVIFSPVIVPAMIAIGLAVTGFLTIGTFGLGGSGG

SEQLEKKLQALEKKLAQLEWKNQALEKKLAQKLAAALEHHHHHH
CZ

Fig. S1 Total peptide sequences of Trx-OLE-ZIP

ProG-OLE-ZIP

GSAMADIGSLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDD
Truncated Protein G (ProG)
ATKTFTVTEKPEVIDASELTPAVTTYKLVINGKTLKGETTTEAVD

AATAEKVFKQYANDNGVDGEWTYDDATKTFTVTEKPEVIDASELT

PAVTTYKLVINGKTLKGETTTKAVDAETAEKAFKQYANDNGVDGV

WTYDDATKTFTVTEGGSGGSMAALKKELQANKKELAQLKWELQAL
NZ
KKELAQGGSGGSFGLTGITLVGTVIGLALATPLFVIFSPVIVPAM
OLE(56-106)
IAIGLAVTGFLTIGTFGLGGSGGSEQLEKKLQALEKKLAQLEWKN
CZ
QALEKKLAQKLAAALEHHHHHH

Fig. S2 Total peptide sequences of ProG-OLE-ZIP

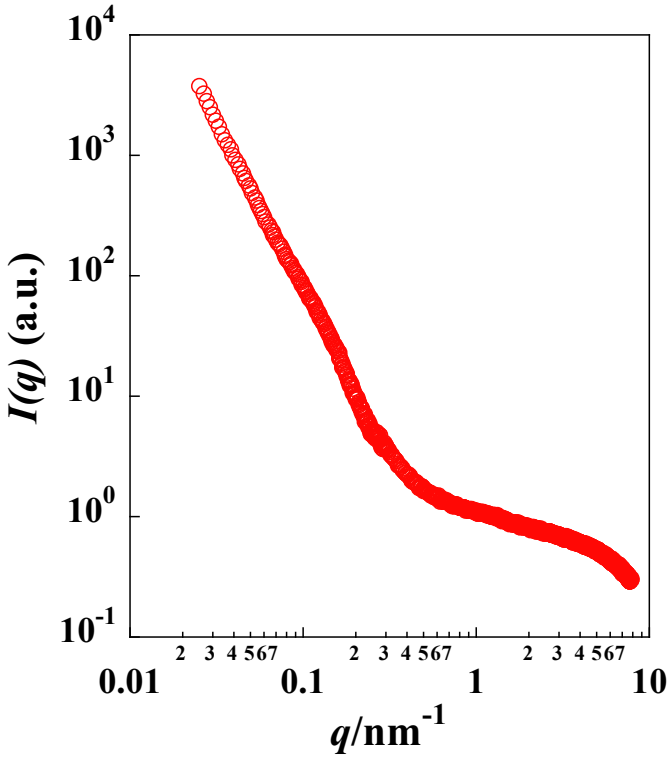

Fig. S3 SAXS analysis of the Trx-OLE-ZIP/ProG-OLE-ZIP protein capsules

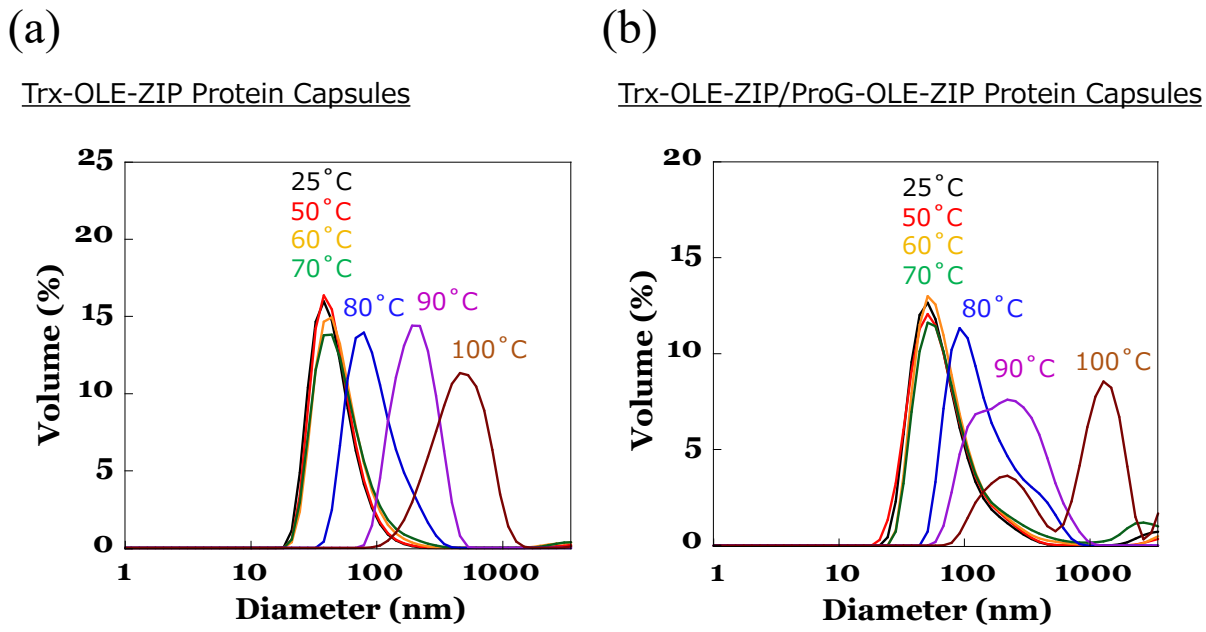

Fig. S4 Temperature dependencies in dynamic light scattering (DLS) profiles of Trx-OLE-ZIP Protein Capsules (a) and Trx-OLE-ZIP/ProG-OLE-ZIP Capsules (b).

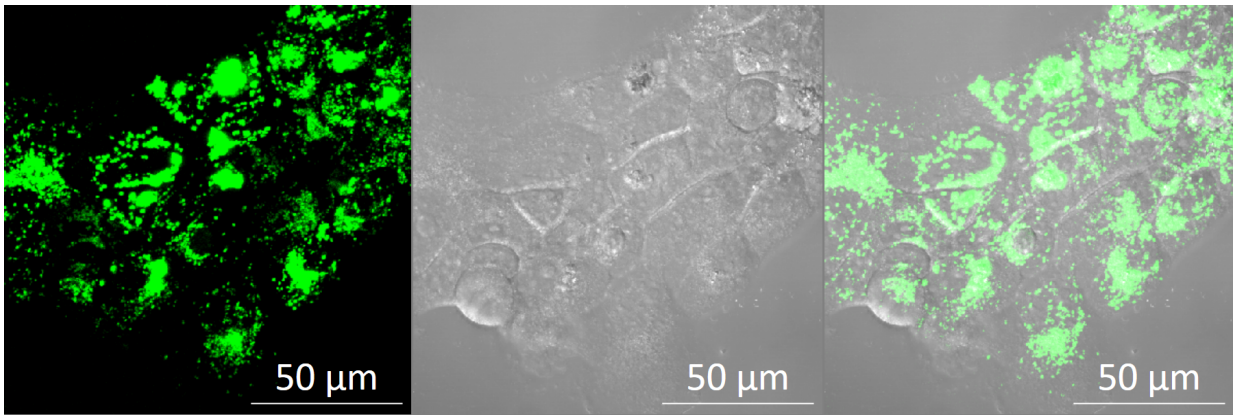

Fig. S5 Confocal Laser Scanning Microscopy (CLSM) observation of A431 cells (1 X 10^5 cells/well) following incubation with Trx-OLE-ZIP/ProG-OLE-ZIP protein capsules (total protein concentration: 1 µM) encapsulating superfolder GFP (sfGFP), supplemented with cetuximab (0.1 µM) for 24 hours at 37 °C. The images include dark field (left panel), bright field (middle panel), and their superimposition (right panel). The green bright spots in the superimposed image correspond to the delivered sfGFP.

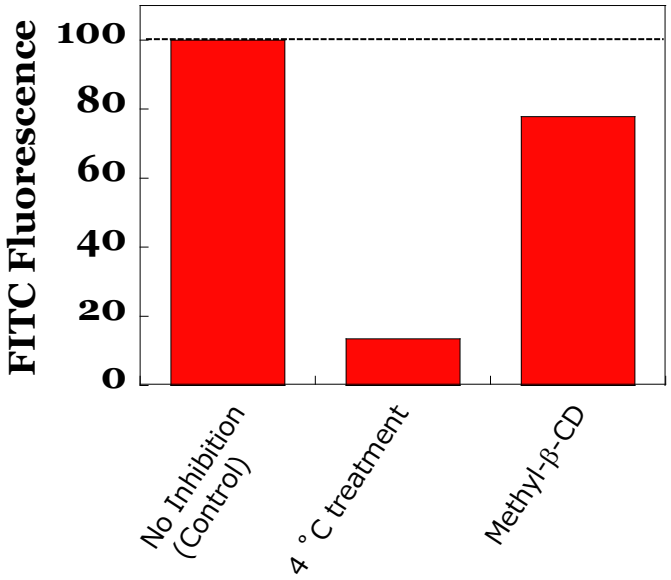

Fig. S6 Impacts of the series of inhibitor addition (methyl-β-CD) or 4˚C treatment on cellular uptake of the Trx-OLE-ZIP/ProG-OLE-ZIP to A431 cells by incubating the Trx-PLE-ZIP/ProG-OLE-ZIP capsules (final capsule protein concentration was 1 µM) with cetuximab (final capsule protein concentration was 0.1 µM) in DMEM with 10% FBS for 24 hr.

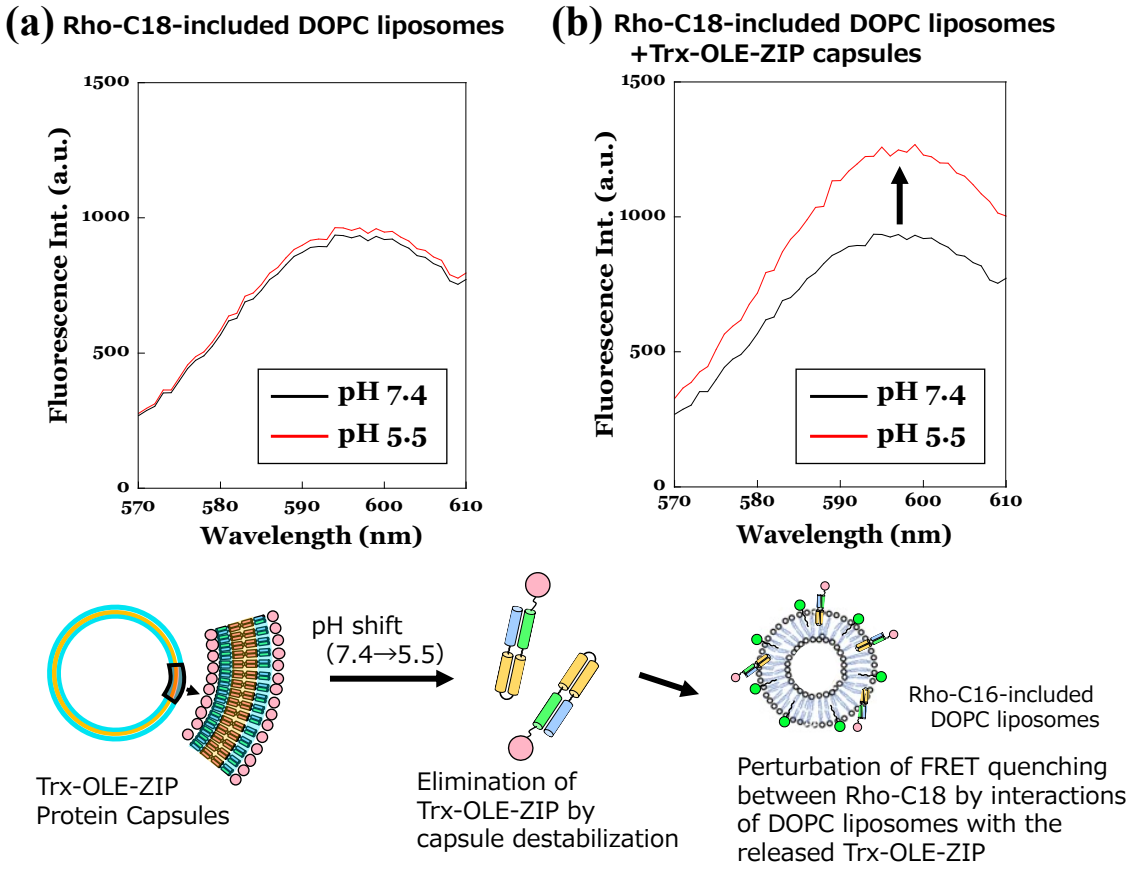

Fig. S7 Alteration of Rho-C18 fluorescence embedded in DOPC liposomes according to pH shift from 7.4 to 5.5 in the (a) absence or (b) presence of the Trx-OLE-ZIP/ProG-OLE-ZIP capsules. (c) Alteration of Rho-C18 fluorescence embedded in DOPC liposomes by surfactant solubilization through triton X-100 addition.

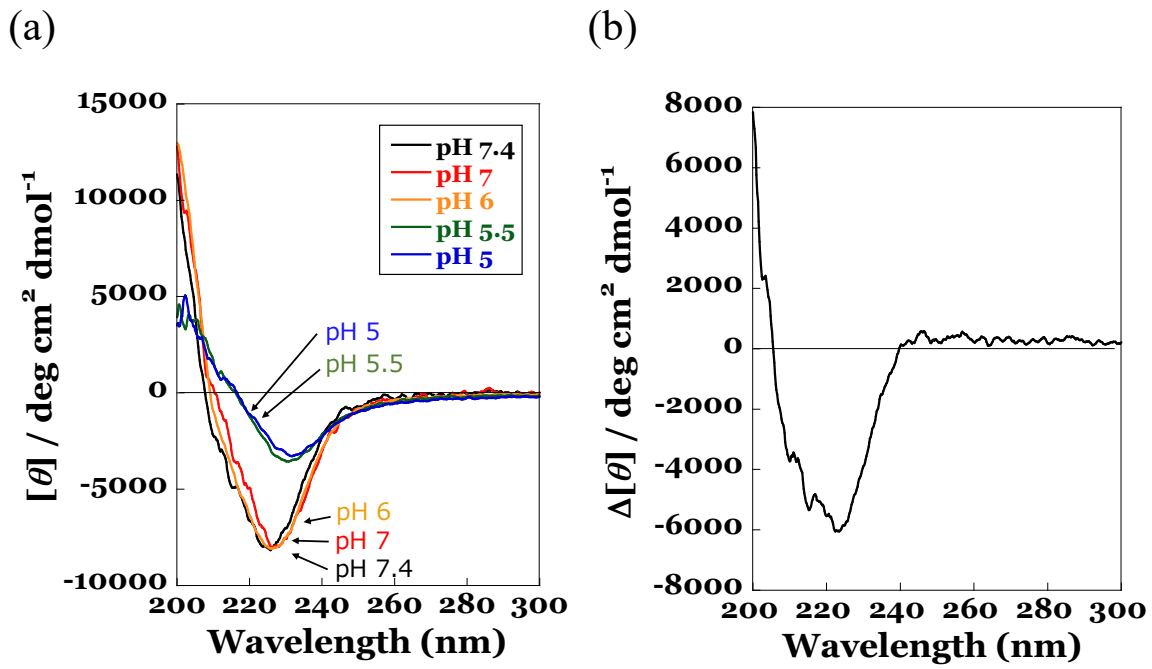

Fig. S8 (a) pH dependence (pH 7.4 to 5) in circular dichroism (CD) spectra of Trx-OLE-ZIP (3 μM) in a 7:93 mixture of 2,2,2-trifuluoroethanol (TFE) and 50 mM phosphate buffer (at pH 7.4, 7, and 6) or 50 mM acetate buffer (at pH 5 and 5.5). (b) Subtracted CD Spectrum of Trx-OLE-ZIP (3 μM) between in a 7:93 mixture of TFE and 50 mM Phosphate Buffer (pH 7.4) and in a 7:93 mixture of TFE and 50 mM acetate Buffer (pH 5).

EXPERIMENTAL

Materials

Restriction enzymes, alkaline phosphatase, and Normal Human Dermal Fibroblasts (NHDF) cell were purchased from Takara-bio. Inc. (Japan). Taq DNA polymerase (AmpliTaq Gold DNA polymerase) was purchased from Thermo Fisher Scientific K.K. (Japan). Rapid DNA Ligation Kit, pET-32a (+) DNA, and fluorescein isothiocyanate (FITC) was purchased from Merck KGaA (Germany). Tris(hydroxymethyl)aminomethane (Tris), isopropyl-β-*D*-thiogalactopyranoside (IPTG), agar, Agarose ME, Ampicillin Sodium, lysogeny broth (LB) medium (Lennox), DMEM, penicillin–streptomycin (100 U/mL), and CCK-8 were purchased from Wako Chemicals (Japan). Sephadex G-50 was purchased from Cytiva (Japan). Oligo DNA*s* for genetic mutations by PCR was purchased from Eurofins Genomics Co Ltd (Japan). Ribonuclease A from Bovine Pancreas was purchased from Nacalai Tesque Inc. (Japan). A431 cell (JCRB0004) was supplied from JCRB cell bank (Japan). Trx-OLE-ZIP was prepared as following the previous study.[1] Other chemicals were used without further purification.

Expression of ProG-OLE-ZIP

A DNA fragment with NcoI and HindIII restriction enzyme sites at each end, encoded for ProG-OLE-ZIP was inserted into a modified pET-32a(+) plasmid vector, which lacked the genes for S-tag and the enterokinase digestion site from the original. The expressed protein became a fusion protein, composed of thioredoxin (Trx) and ProG-OLE-ZIP. The gene of Protein G part was obtained by PCR for the chromosomal DNA of *Streptococcus* G148 strain.[2] *Escherichia coli* BL21(DE3) cells were transformed with the plasmid, and were cultured in LB medium (Lennox) supplemented with ampicillin (100 μg/mL) at 37 °C for 3 h, and for an additional 16 h at 20˚C in the presence of 0.1 mM IPTG. The cells were harvested, resuspended in 50 mM Tris HCl buffer (pH 7) with 100 mM NaCl, and sonicated. The supernatant fraction was subjected to Ni-NTA affinity chromatography to isolate the target fusion protein. Then the purified fusion protein (~10 mg) was further digested by thrombin (0.2 mg) in 20 mL of thrombin buffer (20 mM Tris HCl (pH 8.4), 150 mM NaCl, 20 mM $CaCl_2$) at 4 ˚C overnight and ProG-OLE-ZIP was isolated by Ni-NTA affinity chromatography again. The purity of target protein was analysed by SDS-PAGE analysis.

FITC-labelling of Trx-OLE-ZIP

Referring the previous study,[3] we prepared the FITC-labelled OLE-ZIP. Briefly, 20 mg (0.73 μmol) of Trx-OLE-ZIP was suspended in 10 mL of 50 mM carbonate buffer (pH 10) and kept at 4 ˚C for 30 min. FITC (0.57 mg, 1.46 μmol) was then added to the suspension, which was shaken overnight at 150 rpm using an orbital shaker (CD-100e, EYELATEC, Japan). The FITC-labelled Trx-OLE-ZIP was obtained as an insoluble fraction after solvent exchange to deionized water. The FITC-labelled Trx-OLE-ZIP was purified by washing it thrice in deionized water. Any unreacted FITC was removed and checked by SDS-PAGE before use.

Preparation of protein capsules of Trx-OLE-ZIP and ProG-OLE-ZIP

For preparation of protein capsules of the 9:1 mixture of Trx-OLE-ZIP and ProG-OLE-ZIP, 50 μL of inner aqueous phase solution (50 mM Tris-HCl (pH 7.4), 175 mM glucose, 75 mM sucrose) was added to 500 μL of liquid paraffin and it was sonicated to be a homogeneous emulsion by using an ultrasonic disruptor (UD-211, TOMY SEIKO Co. Ltd., Japan). Then 10 μL of TFE solution of the 9:1 mixture of Trx-OLE-ZIP and ProG-OLE-ZIP (5 mg/mL) was further added and sonicated. The obtained emulsion was piled onto 500 μL of buffer solution (50 mM Tris-HCl (pH 7.4), 250 mM glucose) in 1.5 mL tube and this two-phase solution was applied centrifugation (12000 × *g*) for 15 min at ambient temperature.

DLS analyses of the protein capsules of Trx-OLE-ZIP and ProG-OLE-ZIP

The mean hydrodynamic diameter of protein capsules of Trx-OLE-ZIP and ProG-OLE-ZIP was estimated using a Zetasizer Nano ZS (Malvern Instruments Ltd.)

TEM measurements of spherical morphologies of protein capsules of Trx-OLE-ZIP and ProG-OLE-ZIP

TEM images were obtained with JEM-z2500 (JEOL). All samples were supported by dry-cast of protein solutions on a poval-coated Cu grid. The protein capsule solution was dropped Cu grid, after stand for approximately 5 min at room temperature. Droplets were removed with filter paper and stain by sodium phosphotungstate.

SAXS analysis of the protein capsules of Trx-OLE-ZIP and ProG-OLE-ZIP

X-ray scattering measurements were performed at the SPring-8 BL40B2 beamline. A 25.4 × 28.9 cm^2 photon-counting detector (PILATUS3 S 2M, Dectris, Switzerland) was placed 1.0 or 4.0 m from the sample. The wavelength (λ) of the incident X-rays was 1.0 Å. The OLE-ZIP protein capsule solution in Tris HCl (pH 7) was packed into a quartz capillary cell with a light path length of 2 mm. The incident X-ray wavelength, λ, was fixed at 0.71 c5. The correct path length was determined using silver behenate and the magnitude of the scattering vector [$q = (4\pi/\lambda)\sin\theta/2$] with the scattering angle of θ. The scattering intensities as 2D spectra were transformed into 1D profiles by circular averaging to obtain the data set of the scattering intensity [I(q) and q].

Evaluation of uptake behaviours of the protein capsules to A431 or NHDF cell lines using confocal laser scanning microscope (CLSM)

A431 or NHDF cells (1×10^5 cells/well) were seeded onto a glass-base dish (AGC Techno Glass Co. Ltd., Japan) and incubated in DMEM supplemented with 100 U/mL penicillin–streptomycin solution and 10% FBS overnight under 5% CO_2 at 37 °C. After removing the medium, 1 μM solution of the Trx-OLE-ZIP/ProG-OLE-ZIP protein capsules, supplemented with or without 0.1 μM of cetuximab was added to cells for 12, 24, 48 or 72 h at 37 °C. The FITC-labelled Trx-OLE-ZIP was used for this experiment. After removal of the protein capsules solution, the cells were washed in PBS thrice, re-suspended in DMEM without phenol red, and then subjected to CLSM observation.

Evaluation of uptake behaviours of the protein cages to A431 or NHDF cell lines using fluorescence activated cell sorting (FACS) analysis.

A431 or NHDF cells (5×10^5 cells/well) were seeded onto a 6-well plate and incubated in DMEM supplemented with 100 U/mL penicillin–streptomycin solution and 10% FBS overnight under 5% CO_2 at 37 °C. After removing the medium, 1 μM solution of the Trx-OLE-ZIP/ProG-OLE-ZIP protein capsules, supplemented with or without 0.1 μM of cetuximab was added to cells for 24 h at 37 °C. The FITC-labelled OLE-ZIP was used for this experiment. After removal of the protein capsule solution, the cells were washed in PBS thrice. After detachment of cells using 0.25 w/v% trypsin-1mmol/L EDTA·4Na solution, the cells were collected by centrifuge and washed in PBS thrice. The collected cells were resuspended in PBS with 3% FBS and it was subjected to FACS analyses using FACSVerse (BD Biosciences, USA).

Preparation of RNase A-encapsulated protein capsules of Trx-OLE-ZIP and ProG-OLE-ZIP

For preparation of the protein capsules of the 9:1 mixture of Trx-OLE-ZIP and ProG-OLE-ZIP with RNase A, the inner aqueous phase solution (50 mM Tris-HCl (pH 7.4), 175 mM glucose, 75 mM sucrose) with 3.5 μM of RNase A was used instead of that without RNase A. Preparation process was similar to that without RNase A. For isolation of the protein cages with RNase A, gel permeation chromatography (1 cm (i.d.) × 50 cm (h), Sephadex G-50) was applied using 50 mM Tris-HCl (pH 7.4) with 250 mM glucose for protein cages of Trx-OLE-ZIP and 50 mM Tris-HCl (pH 5) with 250 mM glucose for those of Trx-OLE-D-ZIP.

Evaluation of uptake efficiency of RNase A encapsulating in the protein cages, presenting IgG.

A431 cells (1×10^4 cells/well) were seeded onto a 96-well plate and incubated in DMEM supplemented with 100 U/mL penicillin–streptomycin solution and 10% FBS overnight under 5% CO_2 at 37 °C. After removing the medium, the RNase A-encapsulated Trx-OLE-ZIP/ProG-OLE-ZIP protein capsules (1 μM), supplemented with or without cetuximab (0.1 μM), were added to the cells for 72 h at 37 °C. After removal of the protein cage solution, the cells were washed in PBS thrice and subjected to CCK-8 assay to estimate the number of live cells.

Evaluation of uptake behaviour of superfolder GFP (sfGFP) encapsulating in the protein cages, presenting IgG.

A431 cells (1×10^5 cells/well) were seeded onto a glass-based dish (35 mmϕ, AGC Techno Glass Co., Ltd., Japan) and incubated in DMEM supplemented with 100 U/mL penicillin–streptomycin solution and 10% FBS overnight under 5% CO_2 at 37 °C. After removing the medium, the sfGFP-encapsulated Trx-OLE-ZIP/ProG-OLE-ZIP protein capsules (1 μM), supplemented with cetuximab (0.1 μM), were added to the cells for 24 h at 37 °C. After removal of the protein cage solution, the cells were washed in PBS thrice and subjected to CLSM observation.

Membrane fusion assay using the fluorescent-labelled liposomes and the Trx-OLE-ZIP/ProG-OLE-ZIP protein cages

In a round-base test tube (12 mmφ), DOPC (1.00 mg, 1.27 μmol) and Rhodamine B octadecyl ester perchlorate (Rho-C18) (14.1 ng, 0.0178 μmol) were dissolved in 1 mL of $CHCl_3$ and then the solvent was gently volatilized by N_2 stream to form a lipid thin film onto the inner surface of test tube. The residual solvent was further removed in vacuo. 1 mL of HEPES buffer (5 mM, pH 7.4) was added and

heated at 60 ˚C for 20 min to peel lipid thin membranes from the inner surface of test tube. Then this mixture was applied 20 repeats of vortex-mixing (0.5 s) and liquid N_2 freezing (0.5 s). Again, after being heated at 60 ˚C for 30 s 20 repeats of vortex-mixing (0.5 s) and liquid N_2 freezing (0.5 s) was applied. Further using the nano sizer extrusion kit (T&T Scientific corporation, TN, USA) with 100 nm polycarbonate filter, the liposome diameters were controlled in 100 nm. By subjecting to gel permeation chromatography (Sephadex G-50, with a column that was 10 mm in diameter and 400 mm in height), the Rho-C18-included DOPC liposomes fraction was obtained. A 5 mM HEPES buffer (pH 7.5) was used as the eluent, resulting in the collection of 4 mL of the fraction.

For the membrane fusion experiments, we prepared mixed samples by combining 5 μL of the above isolated liposome fraction solution (pH 7.4, 5 mM HEPES) with 94 μL of the Trx-OLE-ZIP/ProG-OLE-ZIP capsule sample solution (total capsule protein concentration was set at 10 μM, 5 mM HEPES buffer (pH 7.4)) and 1 μL of 3 M acetate buffer (pH 5.5) as a sample at pH 5.5. While, another set of mixed samples was prepared with 5 μL of the isolated liposome solution, 94 μL of the Trx-OLE-ZIP/ProG-OLE-ZIP capsule sample solution (also at a total capsule protein concentration of 10 μM), and 1μL of 5 mM HEPES buffer (pH 7.4) as a sample at pH 7.4. By comparing the fluorescence spectra (570 – 610 nm, lex = 540 nm) of these samples, we evaluated alteration of interactions between DOPC liposomes and the Trx-OLE-ZIP/ProG-OLE-ZIP capsules by pH shift from 7.4 to 5.5. As references, we also prepared the mixture of 5 μL of the above isolated liposome fraction solution (pH 7.4, 5 mM HEPES) and 94 μL 5 mM HEPES buffer (pH 7.4) with 1 μL of 3 M acetate buffer (pH 5.5) or 1μL of 3 M acetate buffer (pH 5.5). By comparing the fluorescence spectra (570 – 610 nm, lex = 540 nm) of these samples, we also evaluated alteration of fluorescence character of Rho-C18 in DOPC liposomes by pH shift from 7.4 to 5.5.

Evaluation of uptake pathway using RNase A as a cargo protein

A431 cells (5×10^5 cells/well) were seeded onto a 6-well plate and incubated in DMEM supplemented with 100 U/mL penicillin–streptomycin solution and 10% FBS overnight at 37 °C under 5% CO_2. To evaluate the impact of 4 ˚C treatment on uptake efficiency, cells were preincubated at 4 ˚C for 1 hour. After removing the medium, a 1 μM solution of Trx-OLE-ZIP/ProG-OLE-ZIP protein capsules, supplemented with 0.1 μM of cetuximab, was added to the cells and incubated at 4 ˚C for an additional 12 hours. The cells were then detached using 0.25% w/v trypsin-1mmol/L EDTA·4Na solution, collected by centrifugation, and washed with PBS three times. The collected cells were resuspended in PBS with 3% FBS and subjected to FACS analysis using FACSVerse (BD Biosciences, USA). To evaluate the impact of methyl-β-cyclodextrin as an inhibitor of membrane fluidity, cells were preincubated with 2 mM methyl-β-cyclodextrin in DMEM supplemented with 100 U/mL penicillin–streptomycin solution and 10% FBS at 37 ˚C for 1 hour. After preincubation, a 1 μM solution of Trx-OLE-ZIP/ProG-OLE-ZIP protein capsules, supplemented with 0.1 μM of cetuximab, was added to the cells and incubated at 37 °C for an additional 24 hours. The cells were then detached using 0.25% w/v trypsin-1mmol/L EDTA·4Na solution, collected by centrifugation, and washed with PBS three times. The collected cells were resuspended in PBS with 3% FBS and subjected to FACS analysis using FACSVerse (BD Biosciences, USA).

Circular dichroism (CD) spectrum of Trx-OLE-ZIP in different pH

The CD spectra of Trx-OLE-ZIP (10 μM) were recorded using a JASCO J-720 spectrometer (Nihon Bunko, Co. Ltd, Japan) in a 4:96 mixture of 2,2,2-trifluoroethanol (TFE) and 50 mM phosphate buffer (at pH 7.4, 7, and 6), as well as 50 mM acetate buffer (at pH 5 and 5.5). The solution samples were contained in a square quartz cell with a light path of 2.0 mm.

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2. C. R. Goward, J. P. Murphy, T. Atkinson and D. A. Barstow, *Biochem J*, 1990, **267**, 171-177.
3. Y. Z. Zhao, X. Q. Tian, M. Zhang, L. Cai, A. Ru, X. T. Shen, X. Jiang, R. R. Jin, L. Zheng, K. Hawkins, S. Charkrabarti, X. K. Li, Q. Lin, W. Z. Yu, S. Ge, C. T. Lu and H. L. Wong, *J Control Release*, 2014, **186**, 22-31.