Construction of Protein Capsules from Novel Amphiphilic Two-Helix Bundle Proteins (GP-ZiPs), designed by Fusing the Transmembrane Region of Glycophorin A with Coiled-Coil Peptides

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Trx-GP-ZIP41

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKL
TrxA

NIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGHMHH

HHHHSSGLVPRGSAMAALKKELQANKKELAQLKWELQALKKELAQESEGASGGSITLIM
NZ GP-41

PGVMAGVIGTMLLMFSPILLISGIVGAIVGPIILTIGGSGGAGESEQLDKKLQALEKKL
CZ

AQLEWKNQALEKKLAQ

Trx-GP-ZIP29

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKL
TrxA

NIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGHMHH

HHHHSSGLVPRGSAMAALKKELQANKKELAQLKWELQALKKELAQESEGAGVMAGVIGT
NZ GP-29

MLLMFSPILLISGIVGAIVGGAGESEQLDKKLQALEKKLAQLEWKNQALEKKLAQ
CZ

Trx-GP-ZIP20

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKL
TrxA

NIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGHMHH

HHHHSSGLVPRGSAMAALKKELQANKKELAQLKWELQALKKELAQESEVIGTMLLMFSP
NZ GP-20

ILLISGIVGESEQLDKKLQALEKKLAQLEWKNQALEKKLAQ
CZ

Fig. S1 Amino-acid sequences of Trx-GP-ZIPs.

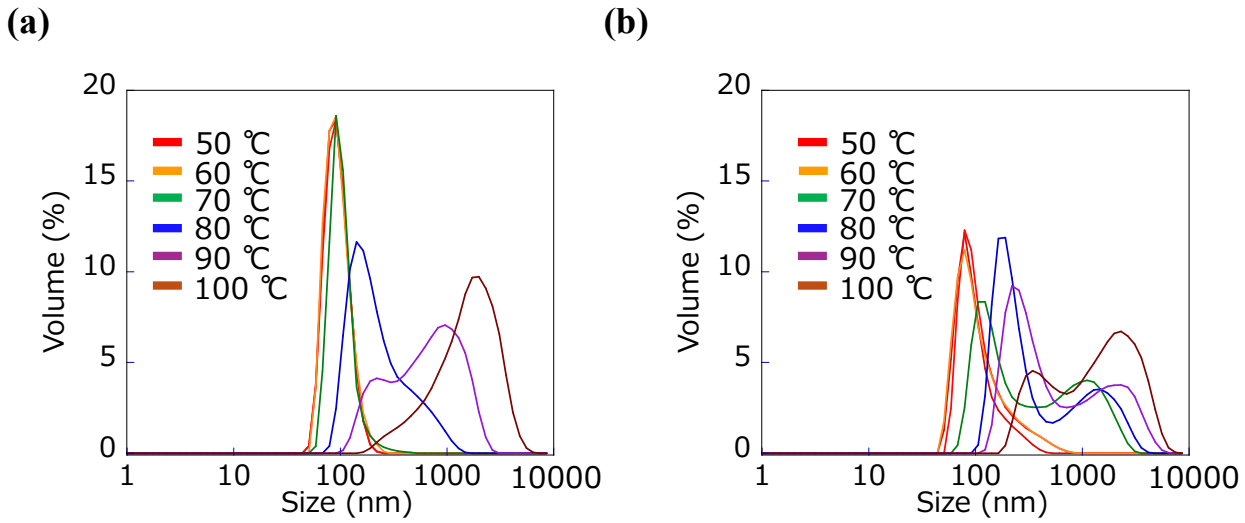

Fig. S2 Alteration of DLS profiles of (a) GP-ZIP 41and (b) GP-ZIP29 capsules after heat treatment of 50, 60, 70, 80, 90, and 100 ˚C.

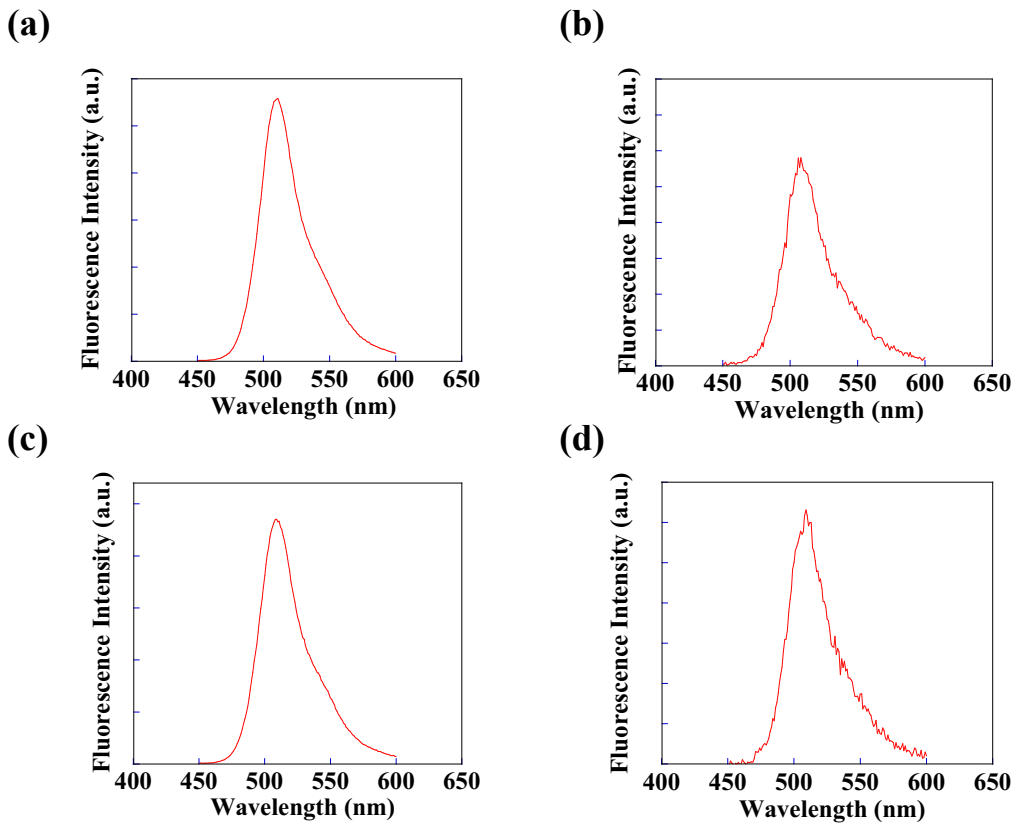

Fig. S3 Fluorescence spectra of GFP: (a) in phosphate buffer (pH 7) and encapsulated within GP-ZIP capsules prepared by (b) Trx-GP-ZIP41, (c) Trx-GP-ZIP29, or (d) Trx-GP-ZIP20.

Experimental

Materials

Restriction enzymes, alkaline phosphatase, TaKaRa Ex Premier DNA Polymerase, DNA Ligation Kit ＜Mighty Mix＞ and His60 Ni Superflow Resin were purchased from Takara-bio. Inc. (Japan). pET-32a (+) DNA and fluorescein isothiocyanate (FITC) were purchased from Merck KGaA (Germany). Tris(hydroxymethyl)aminomethane (Tris), isopropyl-β-*D*-thiogalactopyranoside (IPTG), agar, Agarose ME, Ampicillin Sodium and lysogeny broth (LB) medium (Lennox) were purchased from Wako Chemicals (Japan). Sephadex G-50 was purchased from Cytiva (Japan). Oligo DNA*s* for genetic mutations by PCR and synthetic DNA of GP-ZIP41, GP-ZIP29, GP-ZIP20 were purchased from Eurofins Genomics Co Ltd (Japan). Ribonuclease A from Bovine Pancreas was purchased from Nacalai Tesque Inc. (Japan). Other chemicals were used without further purification.

Expression and purification of Trx-GP-ZIPs

DNA fragments with NcoI and HindIII restriction enzyme sites at each end, encoded for GP-ZIP41, GP-ZIP29, and GP-ZIP20 were 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 A(TrxA) and GP-ZIP41, GP-ZIP29, or GP-ZIP20, respectively. *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 3h at 37˚C in the presence of 1 mM IPTG. The cells were harvested, resuspended in 20 mM Tris HCl buffer (pH 8) with 100 mM NaCl, 10 mM imidazole, and 10 (v/v) % glycerol and sonicated. The pellet obtained by centrifugation (6000 rpm, 5 min, 4 °C) was resuspended in 10 mL of 8 M urea for 3 hours using a shaker (EYELA, Cute Mixer CM-1000) to solubilize the target proteins. The resulting solution was aliquoted into 2 mL microcentrifuge tubes and centrifuged (13,000 rpm, 40 min, 4 °C) to remove any insoluble contaminants. To purify the target proteins in supernatant, the 10 mL protein solution in 8 M urea was mixed with 10 mL of 50 mM phosphate buffer (pH 8.0), and the

mixture was centrifuged to remove host E. coli-derived proteins as a precipitant. The supernatant containing the target protein was transferred into dialysis tubing (pore size: 5 nm; molecular weight cut-off: 12–14 kDa) and dialyzed against 1 L of 2 M urea for 3 hours. Subsequently, stepwise dialysis was performed by gradually decreasing the urea concentration (1 M → 500 mM → 100 mM → 0 M), with each step for 3 hours, followed by overnight dialysis in 0 M urea (pure buffer). The precipitate formed after dialysis was collected as the purified target protein. The purity of target protein was analysed by SDS-PAGE analysis.

FITC-labelling of RNaseA

Referring the previous study,[3] we prepared the FITC-labelled RNase A. Briefly, 20 mg (0.73 μmol) of RNase A 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 RNase A 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-GP-ZIPs with or without encapsulating GFP or FITC-labelled RNase A

For preparation of GP-ZIP protein capsules, 40 μL of inner aqueous phase solution (20 μL of 50 mM phosphate buffer (pH 7) with 150 mM sucrose and 20 μL of TFE were mixed) containing Trx-GP-ZIP41, Trx-GP-ZIP29, or Trx-GP-ZIP20 with or without GFP or FITC-labelled RNase A 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). The obtained emulsion was piled onto 500 μL of 50 mM phosphate buffer in 1.5 mL tube and this two-phase solution was applied centrifugation (12000 × *g*, 4 ˚C, 15 min). The lower aqueous phase was carefully collected and transferred to a new 1.5 mL

microcentrifuge tube. To ensure complete removal of any residual precipitates or liquid paraffin, the sample was centrifuged again at 13,000 rpm for 10 minutes at 4 °C.

DLS analyses of the protein capsules of Trx-GP-ZIPs

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

TEM measurements of spherical morphologies of protein capsules of Trx-GP-ZIPs

TEM images were obtained with JEM-1400 Plus (JEOL, Japan). All samples were supported by dry-cast of protein solutions on a poval-coated Cu grid (Okenshoji Co., Ltd, Japan). The protein capsule solution was dropped Cu grid, after stand for approximately 5 min at room temperature. Droplets were removed with filter paper and dried. TEM analysis was performed at an accelerating voltage of 100 V.

Heat-treatment of Protein capsules of Trx-GP-ZIPs

A 150 μL aliquot of the lower-phase solution prepared by an emulsion procedure was transferred to a 1.5 mL microcentrifuge tube and subjected to heat treatment at various temperatures (50, 60, 70, 80, 90, and 100 °C) for 5 minutes using a heat-block apparatus (MD-MINI, Major Science Co., Ltd, Taiwan). The samples were then cooled on ice for 5 minutes, and changes in particle size were evaluated using a Zetasizer Nano ZS (Malvern Panalytical Ltd., UK).