Electronic Supplementary Material (ESI) for Molecular BioSystems.

Supporting Information

Plasma membrane translocation of a protein needle based on a triple-stranded β-helix motif

Nusrat J. M. Sanghamitra,[a] Hiroshi Inaba,[b] Fumio Arisaka,[c] Dan Ohtan Wang,[a] Shuji Kanamaru,[c] Susumu Kitagawa*[a,b] and Takafumi Ueno*[a,c]

[a]Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan. [b]Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan. [c]Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan.

E-mail: kitagawa@icems.kyoto-u.ac.jp, tueno@bio.titech.ac.jp

Materials and Methods

Unless otherwise stated, all chemicals were purchased from commercial suppliers and used without further purification. Sequence of Fluorescein-labeled R8 (R8_F) is FITC-Acp-RRRRRRRR-$CONH_2$ (Acp: 6-aminocaproic acid). R8_F was purchased from Bex Co., Tokyo, Japan. Cell culture plates were obtained from Iwaki, Tokyo, Japan. β-PN_G18C was expressed and purified as previously described.[1] DMEM (Sigma, D5796) containing 5% FBS, 1.8 mM L-glutamine, 0.9 mM sodium pyruvate, 0.1% sodium bicarbonate, 1% MEM nonessential amino acid solution (Sigma, M7145), 90 U/mL penicillin, and 90 μg/mL streptomycin was used as the cell culture medium. For MALDI-TOF analysis, samples were dialyzed against a 5 mM ammonium acetate aqueous solution and subsequently mixed with an equal volume of a 70% v/v acetonitrile/water solution containing 0.03% w/v sinapic acid and 0.1% v/v trifluoroacetic acid. Confocal measurements were performed using a Nikon Fast Scan Confocal Fluorescence Microscope (Nikon A1R) with 60 × oil objectives. Flow cytometry was performed using a BD FACSCanto™ II flow cytometer with 488 nm laser excitation and a 530/30 band pass filter.

DLS and zeta potential measurements.

DLS measurements were performed using a Zetasizer μV system (Malvern, UK). Three micromolar samples in 0.1 M sodium phosphate, pH 7.0, were analyzed. Zeta potential measurements were performed using a Zetasizer Nano ZSP system (Malvern, UK). Three micromolar samples in 10 mM sodium phosphate, pH 7.4, were analyzed.

Culture of hRBCs.

Human blood was obtained from Veritas (Fresh NPB whole blood collected from healthy volunteers, Lot No. WB001). hRBCs were obtained after centrifugation of a mixture of fresh blood and 0.9% NaCl (1:10) at 1,370 rpm for 3 min. The cells were washed three times with 0.9% NaCl to remove the plasma and buffy coat. The supernatant was removed, and the cells were suspended in a 0.9% NaCl solution. hRBCs were prepared before each experiment and used immediately.

Culture of HeLa cells.

HeLa cells were maintained in cell culture medium at 37°C under 5% CO_2. A subculture was performed every 3-4 days. Cells were grown in 10-cm dishes and incubated at 37°C under 5% CO_2 to approximately 70% confluence. Cells were cleaved by adding a trypsin-EDTA (GIBCO) solution for 5 min at 37°C. Cells were collected by centrifugation at 1,370 rpm for 3 min, and the supernatant was removed.

Characterizations of β-PN_A for penetration into HeLa cells.

Ultracentrifugation and gel permeation chromatography (GPC) analysis were performed to assess whether the needle structure of β-PN_A was maintained in the medium and in cells. Sedimentation velocity analytical ultracentrifugation of β-PN_A in medium exhibited an identical profile as in 0.1 M sodium phosphate, pH 7.0, in which the major peak was assigned to β-PN_A. This result indicated that β-PN_A did not aggregate in the medium prior to penetration (Fig. S7). The GPC elution profile of the cell lysate after 6 h incubation of β-PN_A with HeLa cells exhibited a peak at 12.3 min (Fig. S8), which is identical to that observed for β-PN_A in medium and indicates that the structure of β-PN_A was maintained following uptake into living cells. The intracellular

localization of β-PN_A following internalization was investigated by incubation of β-PN_A with HeLa cells transfected with mammalian expression vectors containing RFP fused with a targeting sequence for the Golgi and the Golgi-ER network. β-PN_A was found to be primarily localized in Golgi bodies after an 8 h incubation (Fig. S9). The cell viability of HeLa cells following uptake of the needles was evaluated using an MTT assay (Fig. S10). HeLa cells were incubated with protein needles for 6 h and for an additional 24 h after washing. The viability of HeLa cells incubated with β-PN_A was not altered in comparison to that of untreated cells, indicating that the uptake of β-PN_A was not detrimental to HeLa cells.

Ultracentrifugation.

Sedimentation velocity analytical ultracentrifugation was performed as previously described.[1] The sedimentation velocity profiles of β-PN_A (1.2 μM) in 0.1 M sodium phosphate, pH 7.0, or DMEM (Sigma, D5921) containing 5% FBS, 1.8 mM L-glutamine, 0.9 mM sodium pyruvate, 0.1% sodium bicarbonate, 1% MEM nonessential amino acid solution (Sigma, M7145), 90 U/mL penicillin, and 90 μg/mL streptomycin were monitored at 496 nm.

GPC assay.

HeLa cells (2×10^5 cells) were plated in 1.5 cm dishes and cultured in cell culture medium at 37°C under 5% CO_2 for 12 h. β-PN_A (0.83 μM) was added to the cells and incubated for 6 h. After washing three times with 1 × PBS, the cells were collected by trypsinization and centrifugation. After removal of the supernatant and washing with 1 × PBS, 10% Triton X-100 was added to lyse the cell membrane. After centrifugation,

the supernatant was used for GPC. GPC was performed using an HPLC system and columns (Asahipak GF-510 HQ, Shodex, Tokyo, Japan) at room temperature and 20 mM Tris-HCl, pH 8.0, as the eluent. The elution of β-PN_A was monitored by fluorescence using excitation and emission wavelengths of 496 nm and 538 nm, respectively.

Colocalization assay.

HeLa cells (2.0×10^4 cells) were plated in 1.5 cm glass-bottom dishes and grown to 70% confluence followed by transfection with mammalian expression vectors containing TagRFP, which was fused with a targeting sequence for the Golgi or Golgi-ER network, using lipofectamine in modified DMEM without serum (Opti-MEM medium). After 1 h incubation, the medium was replaced with medium containing serum. After 7 h incubation, β-PN_A (0.83 μM) was added and incubated for 8 h, and the cells were washed with 1 × HBSS. Imaging was performed without fixing the cells using a Zeiss Two-photon Confocal, FCSS Confocal Microscope (LSM 780) and the filter sets of excitation BP470/40 and emission BP525/50 for ATTO520 and excitation BP545/25 and emission BP605/70 for DsRed.

MTT assay.

HeLa cells (1.0×10^4 cells) were plated in a 96-well plate. After incubation with β-PN_A, β-PN_A_pos, β-PN_A_neg (each at a concentration of 0.83 μM), or gp5_A (1.7 μM) in cell culture medium for 6 h at 37°C under 5% CO_2, the cells were washed with 1 × PBS, and cell culture medium was added. After culturing for 24 h, 10 μL of MTT solution (Sigma, M5655) was added to each well and incubated for 4 h at 37°C

under 5% CO_2. After removal of the solution and washing with 1 × PBS, 200 μL of DMSO was added. The cells were lysed, and formazan crystals were solubilized by pipetting. Then, the OD at 570 nm was measured using a 96-well microplate reader.

Labeling of β-PN with Fluorescein.

β-PN_F: A DMSO solution of Fluorescein-5-maleimide (90 μM) was slowly added to an aqueous solution of β-PN_G18C (5.0 μM in 20 mM potassium phosphate, pH 7.0), and the mixture (final concentration of 5% DMSO) was gently stirred at 25°C for 15 h in the dark. β-PN_F was purified using Sephadex G-25 that was equilibrated with 0.1 M sodium phosphate, pH 7.0. MS (MALDI-TOF): [β-PN_G18C monomer + Fluorescein-5-maleimide]$^+$, calcd.: 15,266; found: 15,282.

Comparison of cellular uptake.

HeLa cells (1×10^5 cells) were plated in 1.5 cm dishes and cultured in cell culture medium at 37°C under 5% CO_2 for 12 h. β-PN_F or R8_F (0.83 μM) was added to the cells and incubated for 1 h at 37°C. The cells were washed and collected by trypsinization and centrifugation followed by suspension in 1 × PBS with 5% FBS. Cellular uptake was analyzed using flow cytometry. Each sample was analyzed three times.

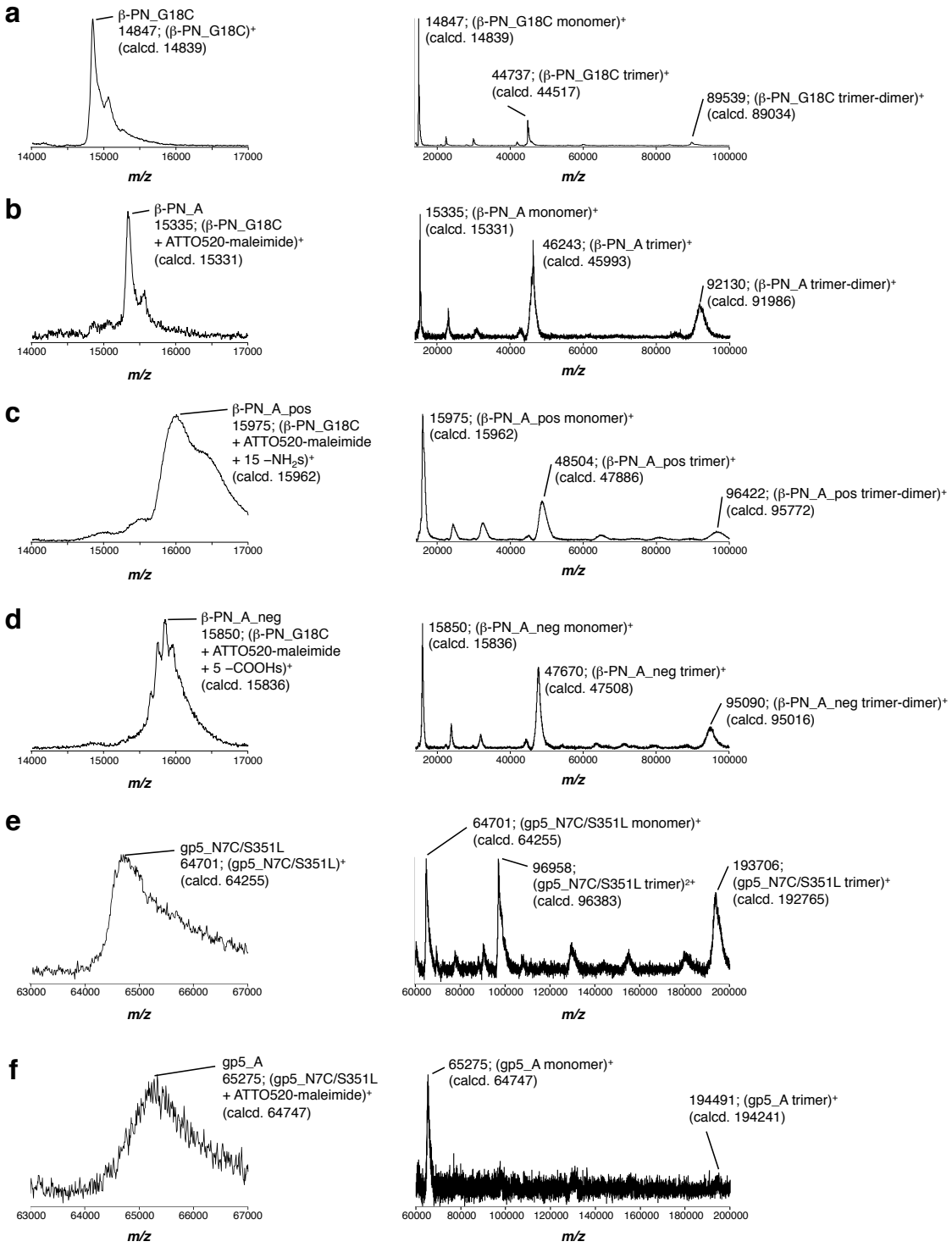

Fig. S1. MALDI-TOF mass spectra of the protein needles.

Monomers (left) and whole structures (right) of each needle. (a) β-PN_G18C, (b) β-PN_A, (c) β-PN_A_pos, (d) β-PN_A_neg, (e) gp5_N7C/S351L, (f) gp5_A.

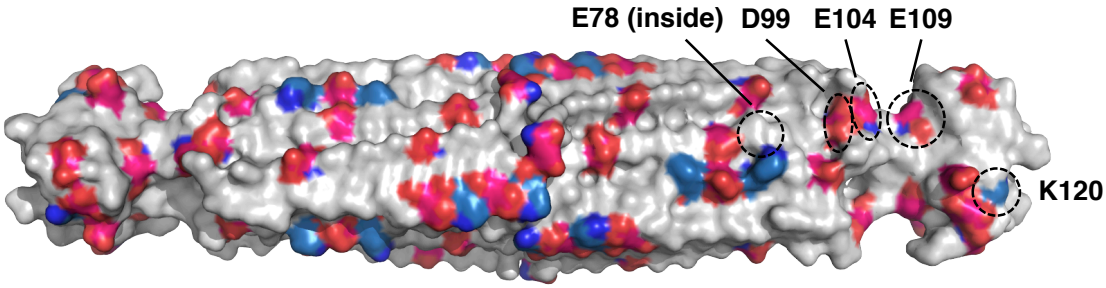

Fig. S2. Surface structure of β-PN.

Asp and Glu, and Lys are shown as red and blue, respectively. Buried residues (E78, D99, E104, E109, K120) are highlighted. E78 is located on the inside of β-PN. One of the six identical residues is shown. The surface structure was prepared using PyMOL (version 0.99rc6).

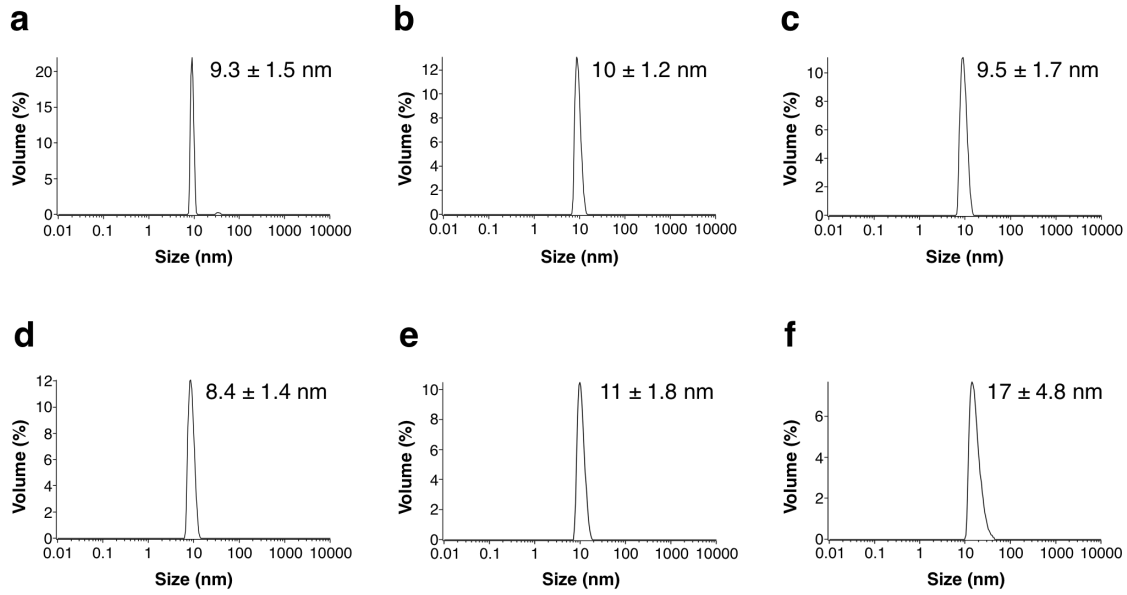

Fig. S3. DLS spectra of the protein needles.

(a) β-PN_G18C, (b) β-PN_A, (c) β-PN_A_pos, (d) β-PN_A_neg, (e) gp5_N7C/S351L, (f) gp5_A. DLS measurements were performed using 3 μM of protein in 0.1 M sodium phosphate, pH 7.0.

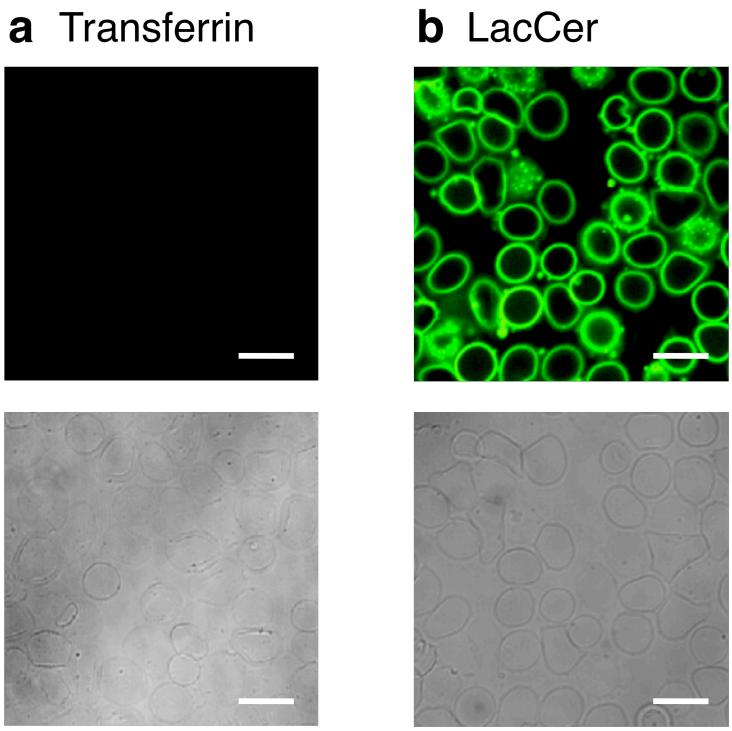

Fig. S4. Confocal images of hRBCs after addition of endocytic markers.

Confocal fluorescence (top) and bright field (bottom) images of hRBCs after addition of (a) human Transferrin (hTf)-AlexaFluor488 (Invitrogen) and (b) BSA-complexed BODIPY FL C5-Lactosylceramind (LacCer) (Invitrogen) (scale bars, 10 μm). The markers (1 μM) were incubated with hRBCs for 1 h in 0.9% NaCl at 37°C under 5% CO_2. Acid wash back exchange protocol was applied to (a) and (b), respectively as described previously.[2]

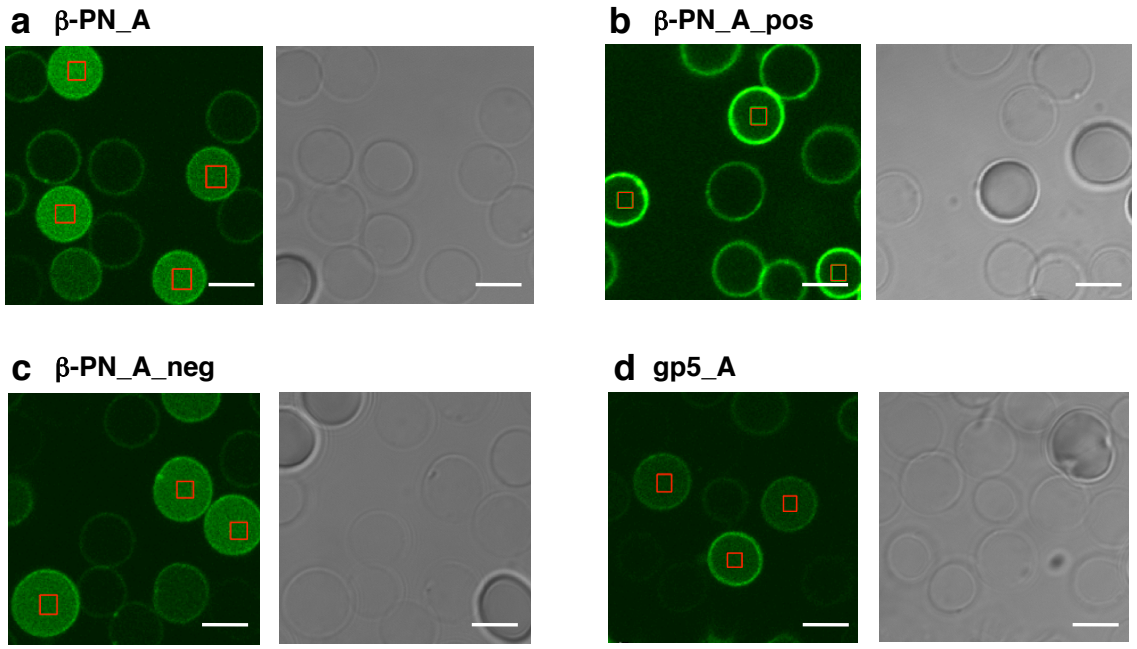

Fig. S5. Calculation of intracellular uptake efficiency in hRBCs.

Confocal fluorescence (left) and bright field (right) images of (a) β-PN_A (b) β-PN_A_pos (c) β-PN_A_neg and (d) gp5_A in hRBCs (scale bars, 5 μm). Each needle was incubated with hRBCs for 1 h in 0.9% NaCl at 37°C under 5% CO_2. The concentration of ATTO520 was adjusted to 10 μM for comparison of the fluorescence intensity. The concentration of β-PN_A, β-PN_A_pos, and β-PN_A_neg was 1.7 μM, whereas that of gp5_A was 3.3 μM. Fluorescence intensity inside of red square normalized to the cellular area was calculated for evaluation of intracellular uptake. Eleven cells were analyzed for each sample to calculate the intensity to obtain the average value and SEM.

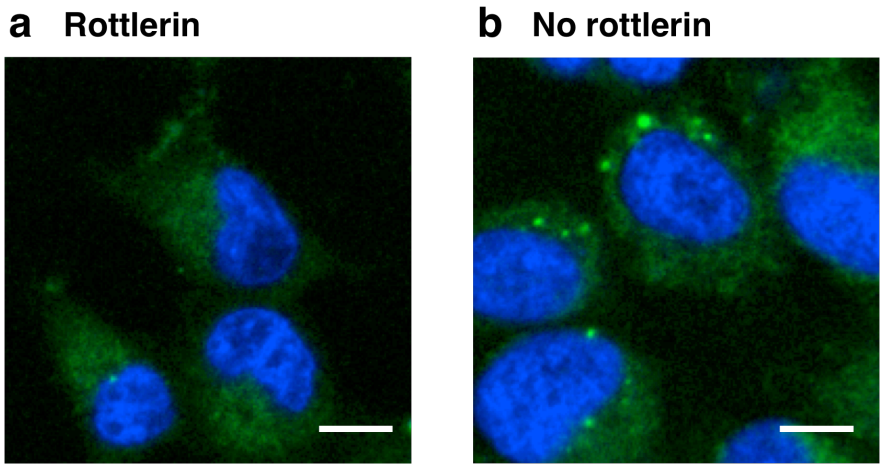

Fig. S6. Uptake of β-PN_A in HeLa cells in the presence of rottlerin.

Confocal fluorescence images of β-PN_A (a) in the presence and (b) in the absence of rottlerin (scale bars, 10 μm). For rottlerin treatment, HeLa cells were preincubated with 10 μM rottlerin for 1 h in cell culture medium without FBS. The cells or untreated cells were washed by PBS and incubated with β-PN_A (1.7 μM) for 1 h in medium at 37°C under 5% CO_2. Cell nuclei were labeled with blue fluorescent Hoechst 33342.

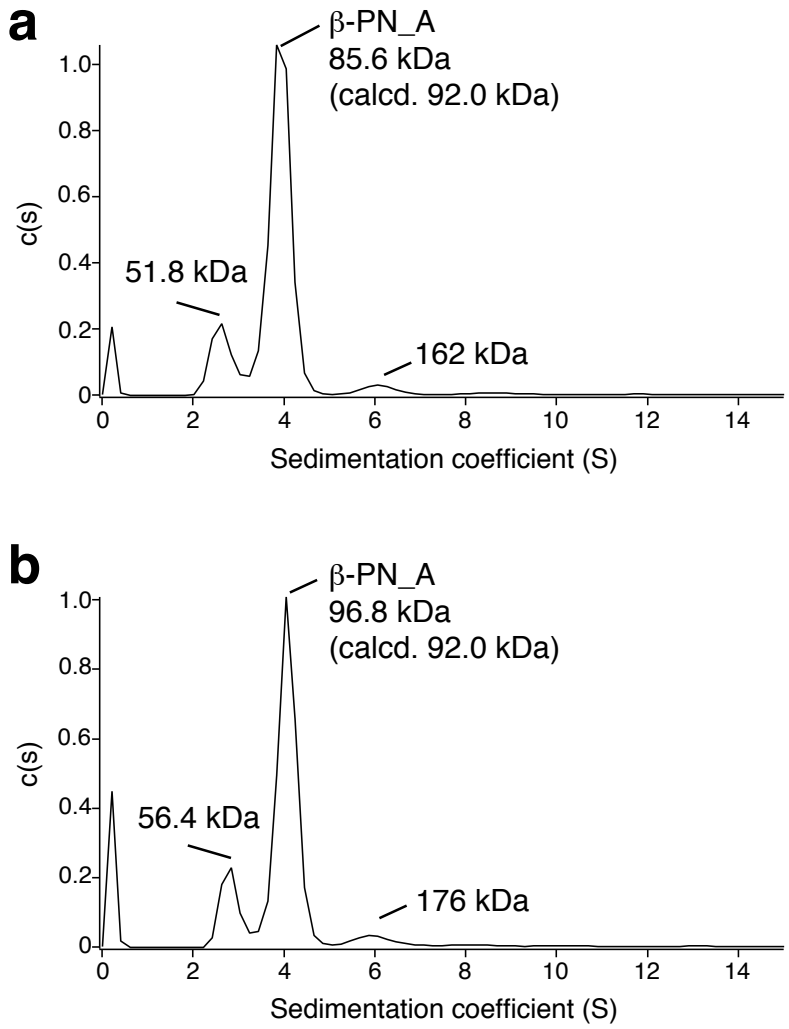

Fig. S7. Sedimentation velocity analytical ultracentrifugation of β-PN_A.

The sedimentation velocity profiles of β-PN_A (1.2 μM) (a) in 0.1 M sodium phosphate, pH 7.0 and (b) in the medium without phenol red were monitored at 496 nm.

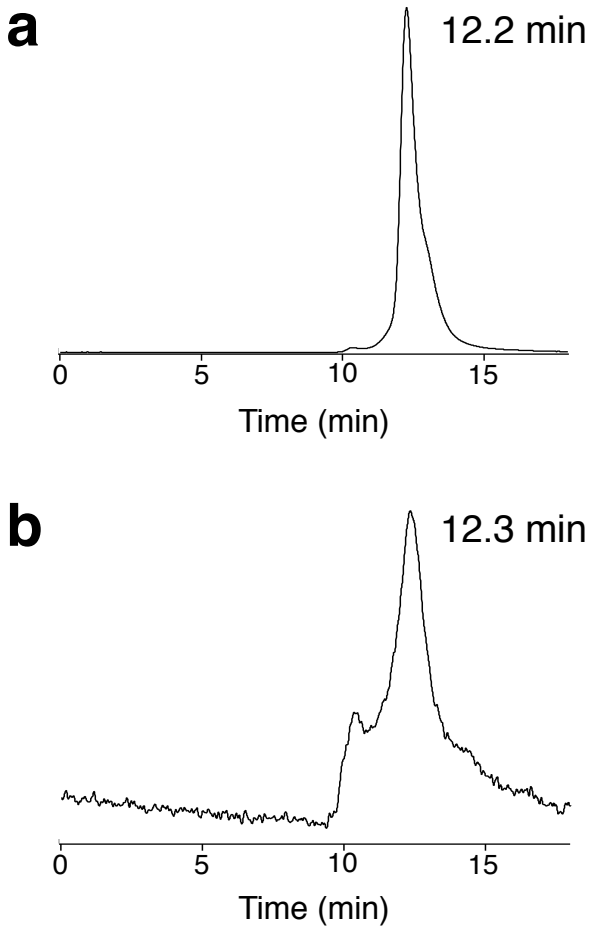

Fig. S8. The elusion profiles of gel permeation chromatography (GPC) of β-PN_A.

The elusion profiles of GPC of (a) β-PN_A in the medium and (b) β-PN_A extracted from HeLa cells. β-PN_A (0.83 μM) was incubated with HeLa cells for 6 h at 37°C under 5% CO_2. β-PN_A was extracted from the cells as explained in Methods. GPC was carried out with a HPLC system and columns (Asahipack GF-510HQ, Shodex, Tokyo, Japan), at room temperature with 20 mM Tris-HCl, pH 8.0 as the eluent. Elution of β-PN_A was monitored by fluorescence. Excitation and emission wavelength were 496 nm and 538 nm, respectively.

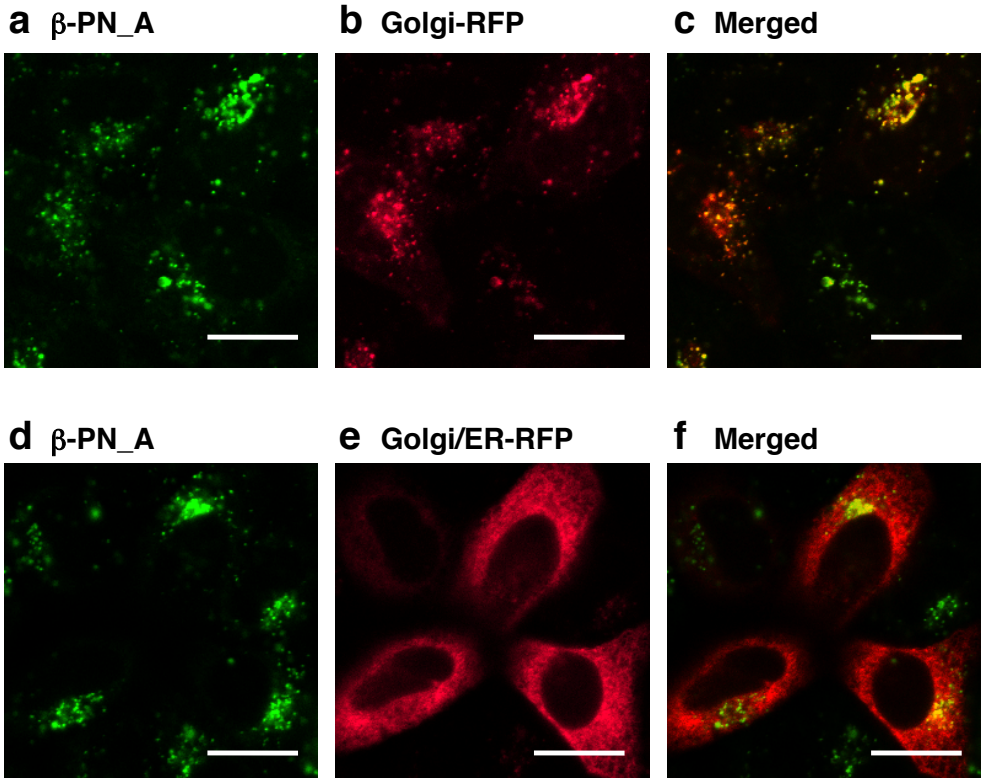

Fig. S9. Colocalization assay of β-PN_A.

Confocal fluorescence images of HeLa cells after incubating with β-PN_A (0.83 μM) for 8 h at 37°C under 5% CO_2 (scale bars, 10 μm). Colocalization of (a)-(c) β-PN_A and Golgi, (d)-(f) β-PN_A and Golgi/ER. The cells were transfected with mammalian expression vectors containing RFP fused with the target sequence of Golgi or Golgi/ER network. The imaging was carried out in Zeiss Two-photon Confocal, FCSS Confocal Microscope (LSM780) by using the filter sets (excitation BP470/40, emission BP525/50) and (excitation BP545/25, emission BP605/70) for β-PN_A (green) and dsRed (red), respectively.

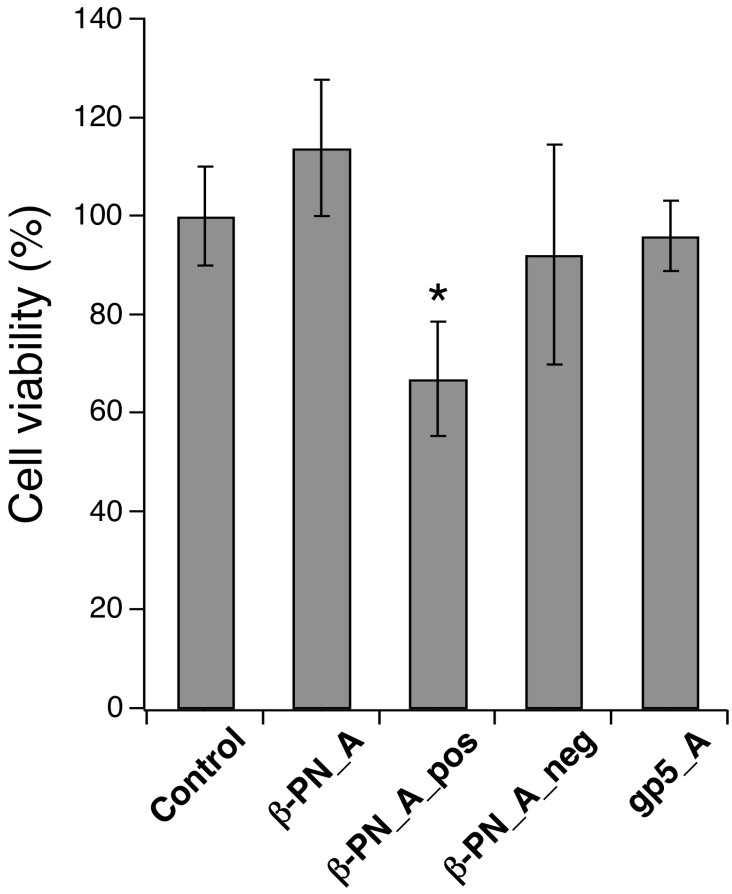

Fig. S10. Viability of HeLa cells in the presence of protein needles.

Cell viability was evaluated using an MTT assay. HeLa cells were incubated with protein needles for 6 h and further 24 h after wash at 37°C under 5% CO_2. Untreated cells were used as a control. The concentration of β-PN_A, β-PN_A_pos, and β-PN_A_neg was 0.83 μM, whereas that of gp5_A was 1.7 μM. The data represent mean ± SEM (n = 3). *P < 0.05 compared to control cells.

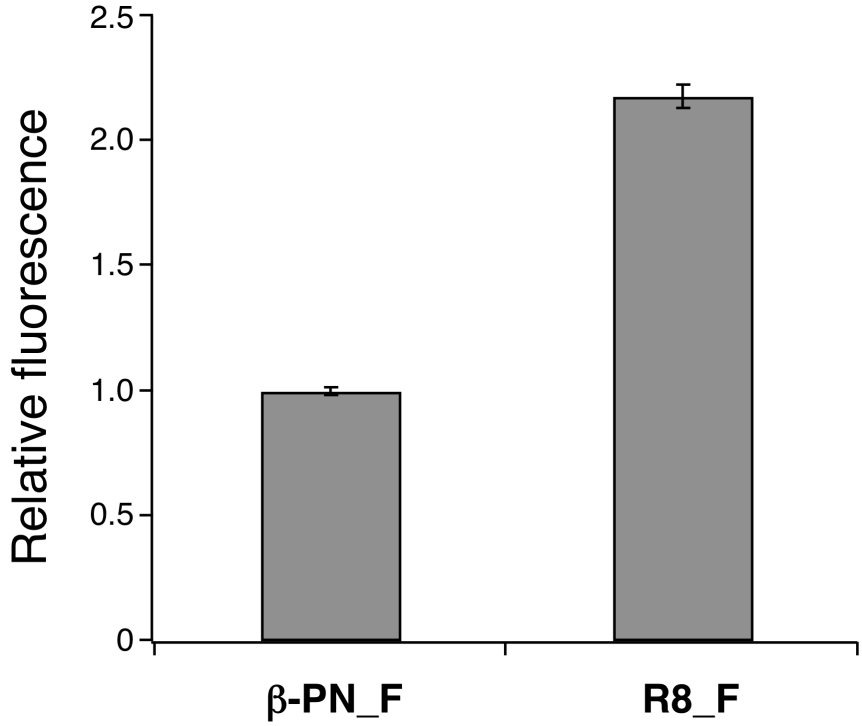

Fig. S11. Comparison of uptake into HeLa cells.

HeLa cells were incubated with β-PN_F or R8_F (0.83 μM) for 1 h at 37°C and analyzed by flow cytometry. The data represent mean ± SEM (n = 3).

References

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2. D. Vercauteren, R. E. Vandenbroucke, A. T. Jones, J. Rejman, J. Demeester, S. C. De Smedt, N. N. Sanders, and K. Braeckmans, *Mol. Ther.*, 2010, **18**, 561–569.