Supplementary information

Affinity-based thermoresponsive fluorescence switching of proteins conjugated with a polymer-binding peptide

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Materials. NovaSynTGR resin, 9-fluorenylmethyloxycarbonyl (Fmoc) amino acid derivatives, and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate were purchased from Novabiochem. 1-Hydroxybenzotriazole monohydrate and *N,N'*-tetramethylenebismaleimide were purchased from Tokyo Chemical Industry Corporation. Human serum albumin (HSA, lyophilized powder, fatty acid free, globulin free, ≥99%), α-cyano-4-hydroxycinnamic acid (CHCA), poly(acrylamide) (M_n = 40000), amino group-terminated PNIPAM (M_n = 5000) and deuterated water were purchased from Sigma-Aldrich. All other reagents were purchased from Nacalai Tesque. Ultrapure water with a resistivity of more than 18.2 MΩ·cm was supplied by a Milli-Q system (Merck Millipore) and was used for all experiments.

Solid-phase peptide synthesis. Peptides with a free *N*-terminus and an amidated *C*-terminus were prepared by solid-phase peptide synthesis using standard Fmoc-based procedures according to a previously published protocol.[1] The peptide chains were assembled on NovaSynTGR resin (amino group 0.25 mmol g^{-1}) using Fmoc amino acid derivatives (3 equiv.) with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (3 equiv.), 1-hydroxybenzotriazole monohydrate (3 equiv.), and *N*,*N*-diisopropylethylamine (6 equiv.) in *N*-methylpyrrolidone (NMP) for coupling and using 20% piperidine in NMP for Fmoc group removal. To cleave the peptides from the resin and to remove the side chain protecting groups, the resins were treated with trifluoroacetic acid (TFA)/thioanisole/*m*-cresol (10/0.75/0.25, v/v/v) for 3 h. The crude peptides were purified by reversed-phase high-performance liquid chromatography (ELITE LaChrom, HITACHI High-Technologies) using a C18 column (COSMOSIL 5C18-AR-300, 20 × 150 mm, Nacalai Tesque) with a linear gradient from 99.9% H_2O/0.1% TFA to 99.9% acetonitrile/0.1% TFA at a flow rate of 6 mL min^{-1}. The peptides were identified by liquid chromatography-mass spectrometry (Prominence UFLC system, MS-2020, Shimadzu) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS, AXIMA-CFR mass spectrometer, Shimadzu) using CHCA as a matrix reagent.

Chemical modification of HSA. HSA with a maleimide group was prepared by reacting *N,N'*-tetramethylenebismaleimide with the Cys34 residue of HSA according to the following procedure. *N,N'*-tetramethylenebismaleimide (15 mM, 50 μL) in DMF was mixed with HSA (170 μM, 450 μL) in phosphate-buffered saline (PBS, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) for 3 d at 37 °C. The products were purified by dialysis (MWCO: 12-14 kDa) in PBS at 4 °C. The amount of unmodified Cys residues in HSA was quantified using Ellman's reagent. HSA (3 μM) was mixed with 5-(3-carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid (10 μM) in PBS for 1 h at ambient temperature. The amount of Cys residues in the HSA was determined by ultraviolet-visible (UV-vis)

spectroscopy (V-550, Jasco). The peptide with an additional Cys residue at the C-terminus (1.7 mM) was reacted with HSA with a maleimide group (170 μM) in PBS (pH 7.4) for 3 d at 37 °C to prepare peptide-modified HSA. The products were purified by dialysis (MWCO: 12-14 kDa) in water at 4 °C and were then lyophilized. The peptide-conjugated HSA was identified by MALDI-TOF-MS using CHCA as the matrix reagent.

The amino groups of the Lys residues of HSA or the peptide-conjugated HSA were labeled with fluorescent molecules. 5-Carboxyfluorescein *N*-succinimidyl ester (10 mM) was reacted with HSA with a maleimide group (150 μM) in PBS for 2 h at 25 °C, and the products were then purified by dialysis (MWCO: 12-14 kDa) in PBS at 4 °C. The number of fluorescein molecules introduced to a single HSA was determined to be 4.6 based on UV-vis absorption spectroscopy (V-550, Jasco). The modification of the fluorescently labeled HSA with peptides was performed according to the aforementioned procedure.

Fluorescence spectroscopy measurements. PNIPAM (50 μg/mL) dissolved in PBS (pH 7.4) containing fluorescently labeled HSA (100 nM) with or without peptide modification was incubated for 20 min at 20 °C and heated to 25 °C at a heating rate of 0.1 °C/min using a thermal cycler. The solutions were incubated for 20 min at 25 °C and then heated to 50 °C at a heating rate of 0.1 °C/min using a thermal cycler. The solutions were incubated for 20 min at 50 °C and were cooled to 25 °C at a cooling rate of 1 °C/min. The solutions were incubated at 25 °C for 20 min. These heating and cooling cycles (heating and cooling rate of 1 °C/min) were repeated 5 times. The fluorescence spectra of the solution after 20 min of incubation at each temperature exited at 496 nm were recorded with a fluorescence spectrophotometer (FP-6500, Jasco). In the case of fluorescence measurements for the Trp residues, the fluorescence spectra were also recorded at an excitation wavelength of 280 nm.

Dynamic light scattering (DLS) measurements. The solutions used for fluorescence measurements were also assessed by DLS measurements. An aliquot (50 μL) of the nanoparticle solution was used for the measurements (Zetasizer Nano ZSP, Malvern) with a micro cell (DTS2145 Low volume glass cuvette, Malvern) at each temperature. The measurements were made after incubation for 5 min in the instrument at each temperature.

Nuclear magnetic resonance (NMR) spectroscopy. ^{1}H NMR spectra of the mixed solution of the PNIPAM-binding peptide (2.5 mM) and PNIPAM (5 mg/mL) dissolved in deuterated PBS were recorded on an AVANCEIII HD500 (Bruker Biospin, 500 MHz) instrument based on 64 scans at 25 °C and referenced to sodium trimethylsilylpropanesulfonate as a standard (δ = 0.0 ppm). As a control experiment, PAM was utilized instead of PNIPAM.

The peptide solution dissolved in deuterated PBS at a concentration of 12.5 mM was used for ^{1}H correlation spectroscopy (COSY), ^{1}H-^{13}C heteronuclear multiple quantum

correlation (HMQC), and ^{1}H-^{13}C heteronuclear multiple-bond correlation (HMBC) spectroscopy at 25 °C (numbers of accumulated measurements were 16, 256, and 256, respectively).

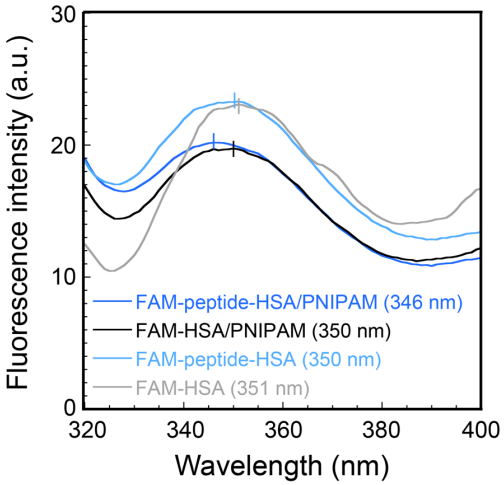

Fig. S1 Fluorescence spectra of FAM-peptide-HSA and FAM-HSA in the presence and absence of PNIPAM exiting the Trp residue (280 nm). The HSA molecules and PNIPAM concentrations were 100 nM and 50 μg/mL, respectively.

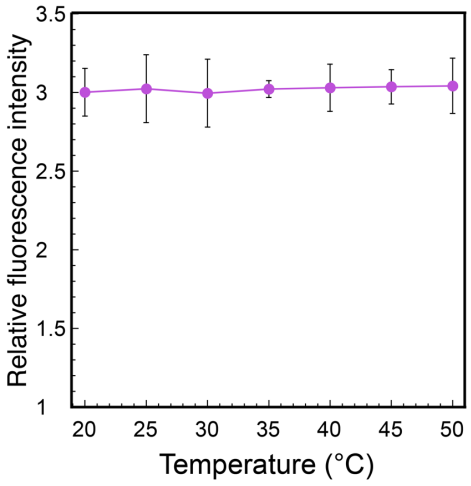

Fig. S2 Fluorescence intensities of FAM-peptide-HSA in the presence of amino group-terminated PNIPAM with a lower molecular weight relative to those in the absence of PNIPAM. The PNIPAM concentration was 50 μg/mL.

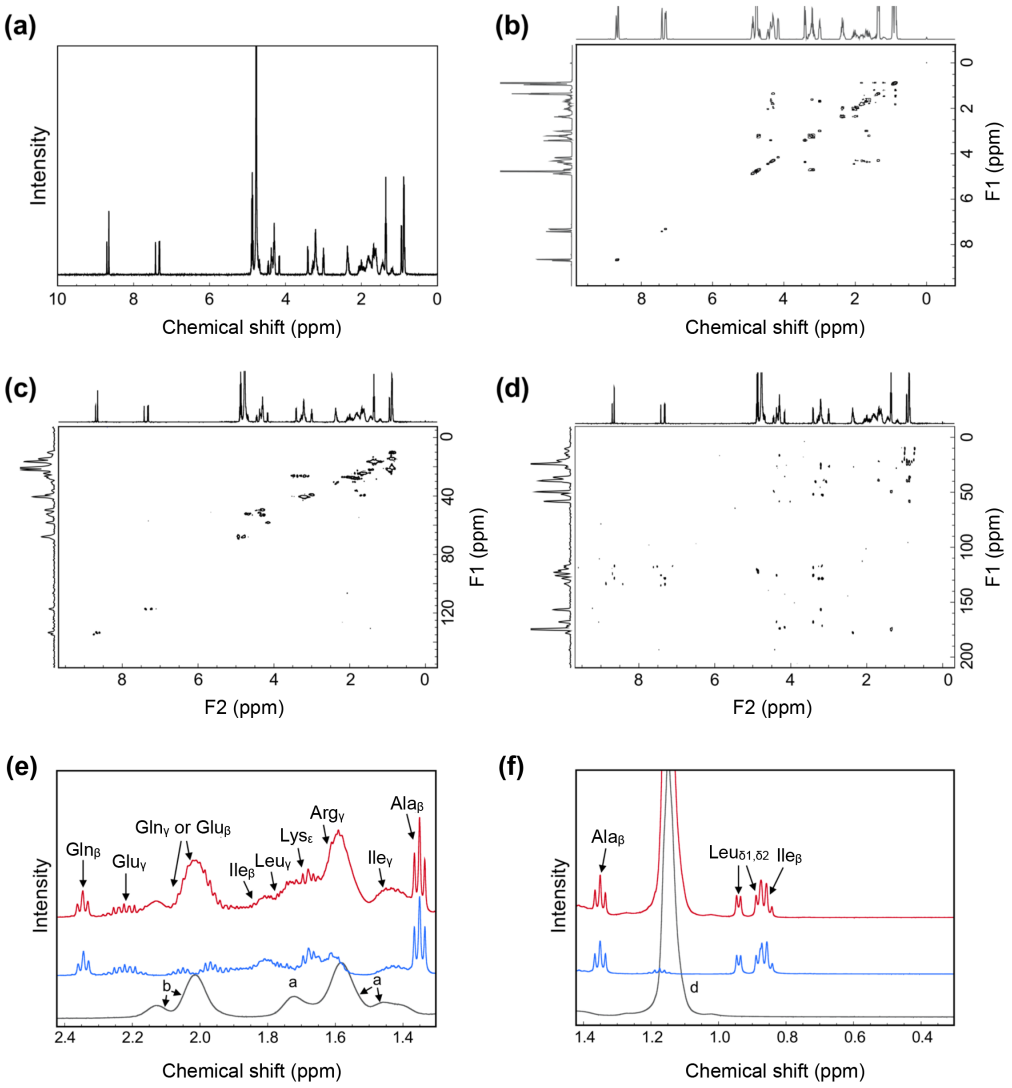

Fig. S3 (a-d) NMR spectra of the peptide. (a) 1D ^{1}H NMR, (b) ^{1}H-^{1}H COSY, (c) ^{1}H-^{13}C HMQC, and (d) ^{1}H-^{13}C HMBC spectra of the peptide. The concentration of peptide was 12.5 mM. (e, f) 1D ^{1}H NMR spectra of the peptide in the presence of PNIPAM (red), the peptide (blue), and PNIPAM (gray) in different chemical shift ranges. The concentrations of the peptide and PNIPAM were 2.5 mM and 5.0 mg/mL, respectively.

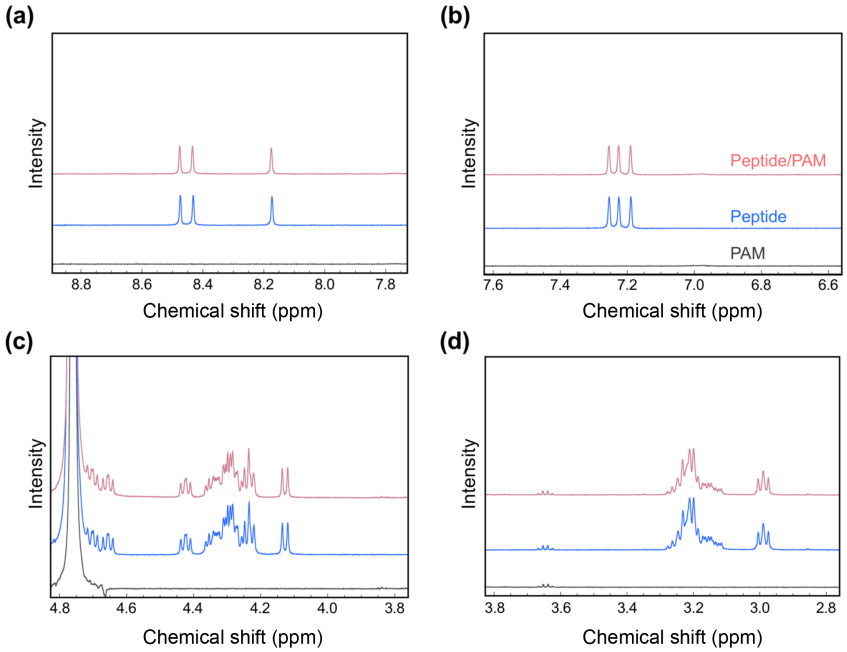

Fig. S4 (a-d) 1D ^{1}H NMR spectra of the peptide in the presence of PAM (red), the peptide (blue), and PAM (gray) in different chemical shifts ranges. The concentrations of the peptide and PAM were 2.5 mM and 5.0 mg/mL, respectively.

References

1 W. C. Chan and P. D. White, in Fmoc Solid Phase Peptide Synthesis eds. W. C. Chan and P. D. White, Oxford University: New York, 1st edn., 2000, pp. 41-76.