Electronic Supplementary Information

Affinity-based thermoresponsive precipitation of proteins modified with polymer-binding peptides

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Experimental details

Materials. A Ph.D-12™ Phage Display Peptide Library Kit and *Escherichia coli* strain ER2738 were purchased from New England Biolabs. 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. Bovine serum albumin was purchased from Wako Pure Chemical Industries. Human serum albumin (HSA, lyophilized powder, fatty acid free, globulin free, ≥99%) and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma-Aldrich. All other reagents were purchased from Nacalai Tesque. Ultrapure water with more than 18.2 MΩ·cm was supplied by a Milli-Q system (Merck Millipore) and was used for all the experiments.

Peptide screening against PNIPAM films. The target films composed of water-insoluble poly(*N*-isopropylacrylamide) (PNIPAM, M_n = 11 000, M_w/M_n = 1.20, m : r = 85 : 15) synthesized by living anionic polymerization[1] were prepared by spin-casting (2000 rpm, 1 min) from a *N*,*N*-dimethylformamide (DMF) solution at a polymer concentration of 20 mg mL^{-1} on a glass substrate. Then, the resulting films were vacuum dried for 5 h. Affinity-based peptide screening against the PNIPAM films was performed according to our previously published protocols.[2,3] An aliquot (5 μL) containing 1.2×10^{10} plaque-forming units (pfu) of the phage library solution in Tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.5) was mounted on the target films for 10 min at 30 °C. The films were previously conditioned with TBS for 12 h at 30 °C before mounting the phage library solution. Unbound and weakly bound phages were removed by rinsing the films once using 100 μL of TBS containing 0.1% Tween 20 and then five times using 200 μL of TBS. The bound phages were eluted by mounting 100 μL of the elution buffer solution (0.5 M glycine-HCl containing 1 mg mL^{-1} bovine serum albumin, pH 2.2) on the films for 15 min at 30 °C. The solution containing the eluted phages was neutralized with 5.7 μL of a Tris-buffer solution (1 M Tris, pH 9.1). The eluted phages were amplified by infection with *Escherichia coli* strain ER2738, and the amplified phages were then purified using a polyethylene glycol/NaCl solution for use in the next round of peptide screening. Five rounds of peptide screening were repeated, followed by cloning and DNA sequencing of the phages.

Phage binding assay. The binding capabilities of the screened and wild type phages against the target PNIPAM films were evaluated by titer counting assays. The PNIPAM films were prepared on a 96-well glass plate and were pre-conditioned with TBS for 12 h before

performing the binding assay. Phage solutions (10 pM in TBS, 50 μL/well) were mounted on the films for 1 h at 20 or 30 °C. After rinsing the samples four times with TBS containing 0.1% Tween 20 (100 μL/well) and then once with TBS (200 μL/well) at 20 or 30 °C, the bound phages were eluted using the elution buffer solution (100 μL/well) for 15 min at 20 or 30 °C. The eluted phage solutions were neutralized using a Tris-buffer solution, and the amounts of phages in the solutions were determined by titer counting assays.

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.[4] The peptide chains were assembled on a 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 reverse-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.

Preparation of peptide-modified 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-buffer 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 of 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 of HSA was determined by ultraviolet-visible (UV-vis) spectra (V-550, Jasco). The c2 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-

modified HSA was identified by MALDI-TOF-MS spectrometry using CHCA as a matrix reagent.

Preparation of fluorescently labeled peptide-modified HSA. To quantify HSA after the thermoresponsive precipitation experiments, the amino groups of the Lys residues of 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 spectra (V-550, Jasco). Modification of the fluorescently labeled HSA with peptides was performed according to the aforementioned procedure.

SPR measurements. The films composed of the target PNIPAM and isotactic poly(methyl methacrylate) (it-PMMA, M_n = 35 500, M_w/M_n = 1.12, *mm* : *mr* : *rr* = 98 : 2 : 0, synthesized following conventional living anionic polymerization[5]) as a reference polymer were applied for SPR measurements using a Biacore X (GE healthcare) according to our previously published protocol.[6] The PNIPAM and it-PMMA films with a thickness of approximately 15 and 10 nm, respectively, were prepared on gold-coated glass slides (SIA Kit Au, GE Healthcare) by spin-casting (2000 rpm, 1 min). HBS-N (10 mM HEPES, 150 mM NaCl; pH 7.4, GE Healthcare) was flowed at a rate of 20 μL min^{-1} at 20 °C. After stabilization of the baselines by the HBS-N flow, the peptide solutions in HBS-N were applied to the films during 0 - 180 s (association), and then the peptide-free HBS-N was flowed during 180 - 1000 s (dissociation). The resulting sensorgrams at four peptide concentrations were analyzed using the global fitting of BIAevaluation software version 4.1. The chi-square (χ^2) values were evaluated to be 1.3, 1.8, and 0.7 (an index of fitting reliability) for the c2 peptide against the PNIPAM films, the c2 peptide against the it-PMMA films, and the inverted c2 peptide against the PNIPAM films, respectively. The χ^2 values of less than 10 are considered to be acceptable according to the BIAevaluation handbook. In the case of the SPR measurements of peptide-modified HSA or native HSA against the PNIPAM films, the association (k_1) and dissociation (k_{-1}) rate constants were determined by plotting the observed rate constant (k_{obs}) using the following linear function: $k_{obs} = k_1C + k_{-1}$, where C represents the protein concentration.

Lower critical solution temperature (LCST) measurements. Two PNIPAMs with different meso diad content (meso-rich PNIPAM: M_n = 7 600, M_w/M_n = 1.15, m : r = 58 : 42; meso-poor PNIPAM: M_n = 13 000, M_w/M_n = 1.12, m : r = 17 : 83) synthesized by living anionic polymerizations[1] were used for the LCST measurements. Aqueous PNIPAM solutions (0.5 mg mL^{-1}) with or without 880 μM of peptides were incubated for 1 h at 20 °C.

The turbidity of the solutions derived from transmittance at 500 nm using a 2 mm-thick quartz cell was monitored using a UV-vis spectrophotometer (V-550, Jasco) during heating of the solutions from 20 °C to 50 °C at a heating rate of 0.1 °C min^{-1} without stirring. The LCSTs were determined as the temperature of 50% transmittance.

Circular dichroism (CD) spectra. The CD spectra of the peptide-modified HSA and native HSA dissolved in phosphate-buffer solutions (10 mM phosphate, pH 7.5) at a concentration of 1.7 μM were recorded on a CD spectrometer (J-725, JASCO) under a N_2 atmosphere at 20 °C using a quartz cell with a thickness of 0.2 cm. The data represents the average of four scans in the wavelength range of 190-260 nm with a resolution of 0.5 nm and a scanning speed of 50 nm min^{-1}.

Thermoresponsive precipitation of HSA with PNIPAM. Aqueous solutions of meso-rich PNIPAM (2.0 mg mL^{-1}, 100 μL) containing fluorescently labeled HSA (100 nM) with or without peptide modification were incubated for 20 min at 20 °C and were heated to 50 °C at a heating rate of 0.1 °C min^{-1} using a thermal cycler (PC-320, ASTEC). The heated solutions were incubated for 30 min at 50 °C and were centrifuged (12100 *g*, 30 min) at 50 °C. Then, 15 μL of the supernatants were diluted by 5 times. The fluorescence intensities at 525 nm (peptide-modified HSA) or 521 nm (non-modified HSA) excited at 496 nm were recorded on a fluorescent spectrophotometer (FP-6500, Jasco) at 25 °C to quantify the amount of HSA in the supernatants.

Table S1 The LCSTs of PNIPAMs in the presence or absence of peptides

PNIPAM	LCST (°C)[a]		
	C2 peptide	Inverted c2 peptide	Peptide (-)
Meso-rich	36.2 ± 0.0	38.3 ± 0.1	38.3 ± 0.2
Meso-poor	38.3 ± 0.1	38.1 ± 0.1	38.1 ± 0.0

[a]LCSTs and their standard deviations were obtained from triplicate experiments.

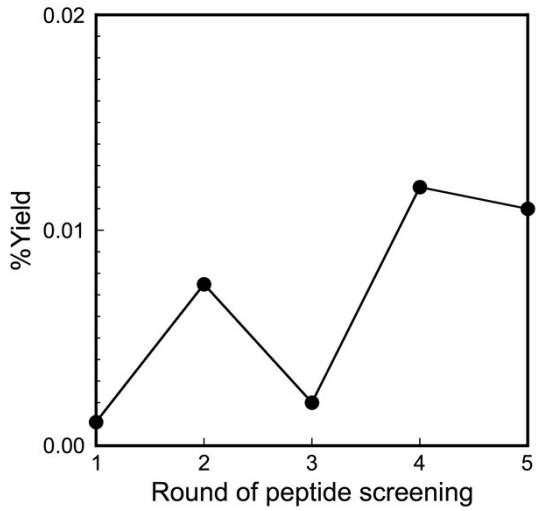

Fig. S1 Percent yields (input/output phages) against the rounds of peptide screening.

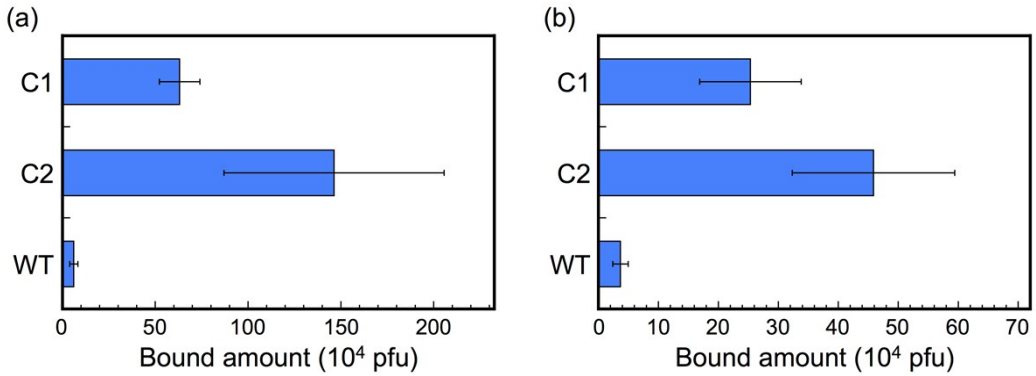

Fig. S2 Bound amounts of the screened phages against the PNIPAM films at (a) 20 °C and (b) 30 °C. Bound amounts and their standard deviations were obtained from triplicate experiments.

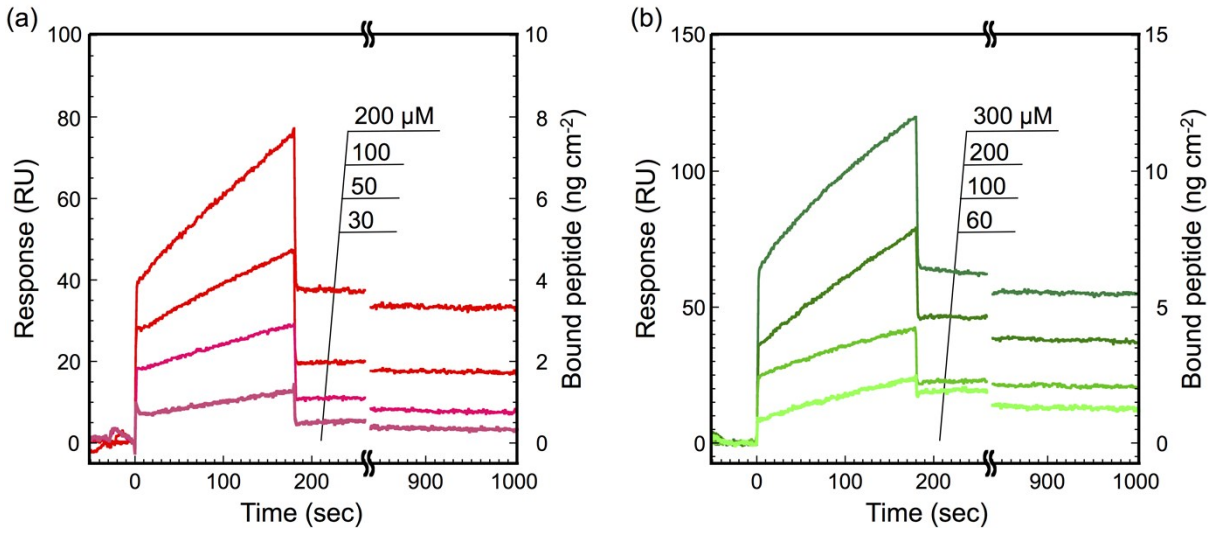

Fig. S3 SPR sensorgrams for the binding of (a) the c2 peptide to the it-PMMA films, and (b) the inverted c2 peptide to the target PNIPAM films at different peptide concentrations.

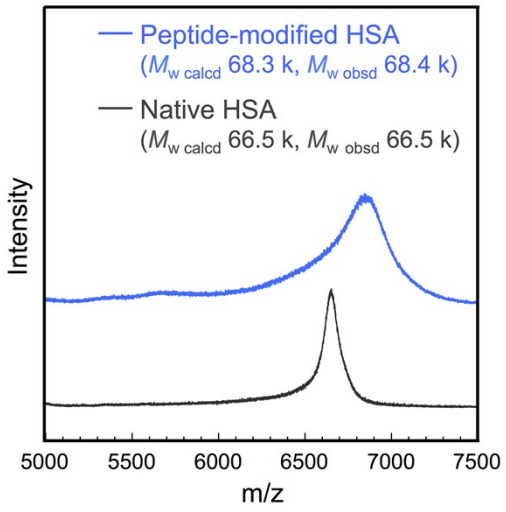

Fig. S4 MALDI-TOF-MS spectra of the peptide-modified HSA and native HSA.

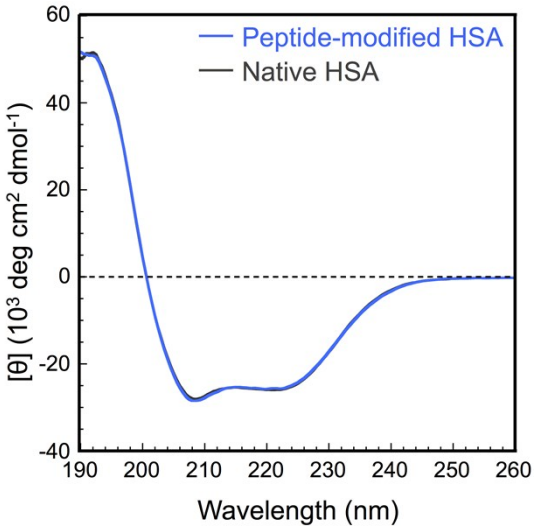

Fig. S5 CD spectra of the peptide-modified HSA and native HSA.

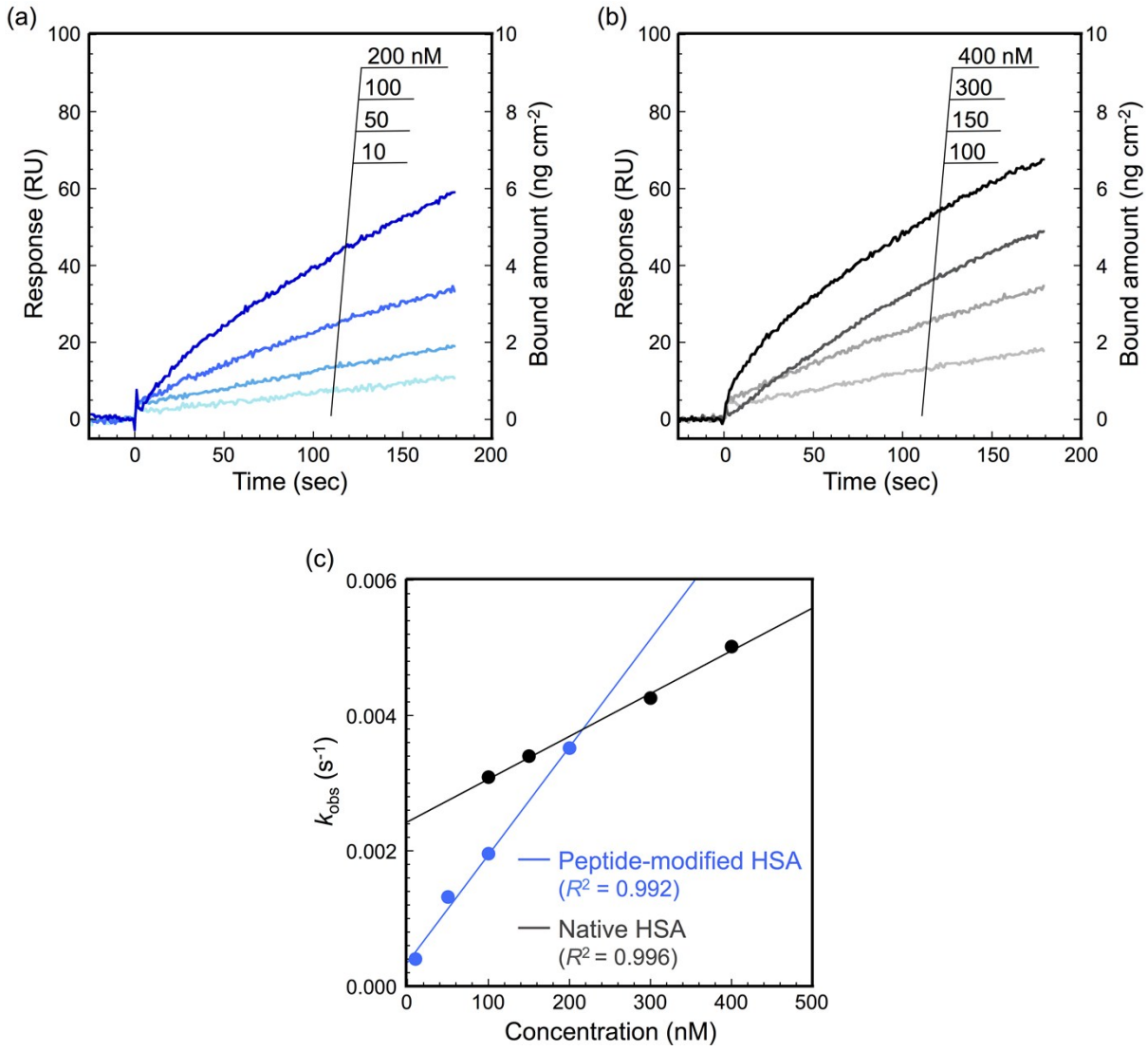

Fig. S6 SPR sensorgrams for the binding of (a) peptide-modified HSA and (b) native HSA to the target PNIPAM films at different protein concentrations. (c) Plots of the k_{obs} of peptide-modified HSA and native HSA for the PNIPAM films as a function of the protein concentration.

Table S2. Kinetic parameters for binding of peptide-modified HSA and native HSA to the PNIPAM films

Protein	k_1 (10^3 M^{-1} s^{-1})	k_{-1} (10^{-4} s^{-1})	K_a (10^7 M^{-1})
Peptide-modified HSA	16	3.7	4.4
Native HSA	6.3	24	0.26

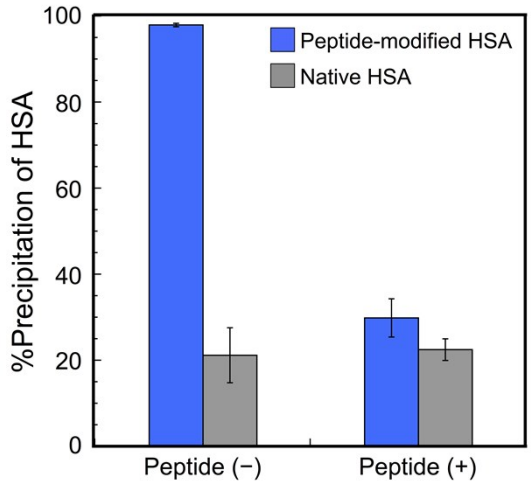

Fig. S7 Percent precipitation of the peptide-modified HSA in the presence or absence of 50 μM free peptides.

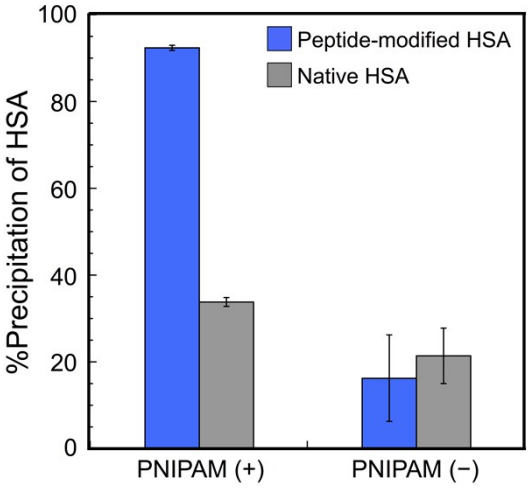

Fig. S8 Percent precipitation of the peptide-modified HSA at an HSA concentration of 10 nM.

References

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