

Supporting information for:

Construction of dye-stapled Quenchbody by photochemical crosslinking to antibody nucleotide binding site

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Methods

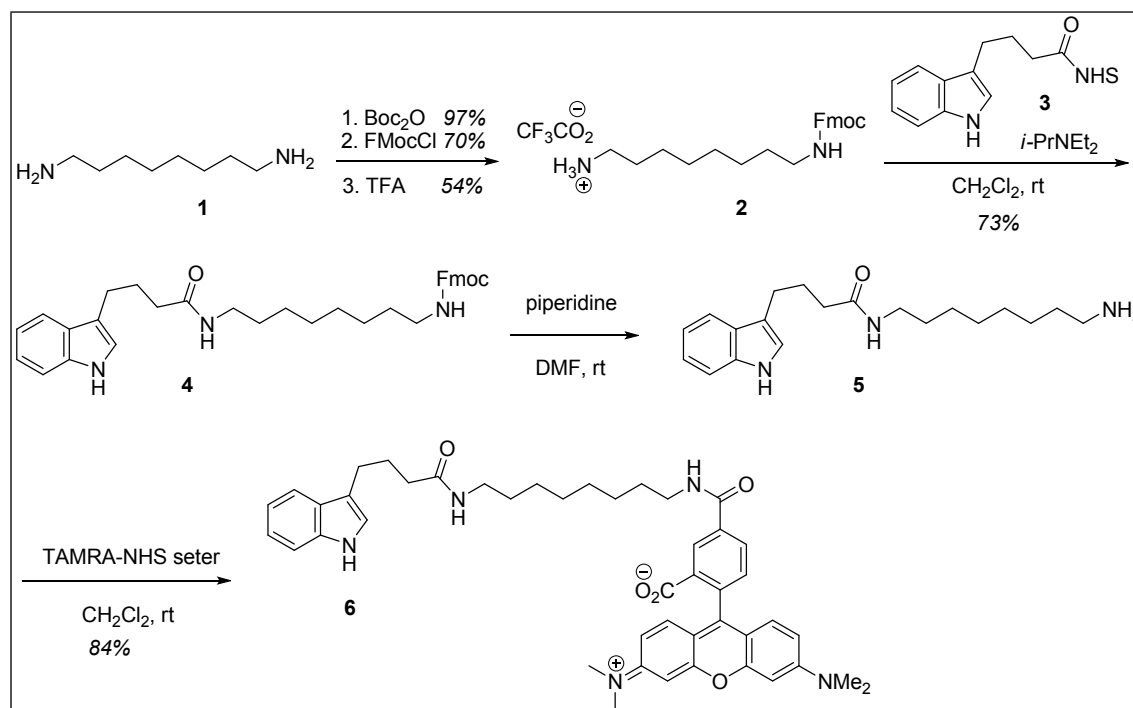
Materials. *E. coli* SHuffle T7 Express lysY was from New England Biolabs (Tokyo, Japan). Talon metal affinity resin and Talon disposable gravity column were from Takara Bio (Shiga, Japan). Ultrafiltration device (centrifugal filter tube Ultra-4, MWCO 3 k) was from Millipore (Tokyo, Japan). Immobilized TCEP disulfide reducing gel was from Thermo Pierce (Rockford, IL). His Sepharose Ni was from GE healthcare (Piscataway, NJ). Anti DYKDDDDK tag antibody beads, DYKDDDDK Peptide, Supersep PAGE gel, silver staining kit, and human serum albumin (HSA) were from Wako pure chemicals (Osaka, Japan). Protein G magnetic beads and 7 k-desalting spin column were from Thermo Pierce (Rockford, IL). Nanosep Centrifugal-3 k Ultrafiltration Device was from Pall (Ann Arbor, MI). Osteocalcin C-terminal peptide (BGP-C7, $\text{NH}_2\text{-RRFYGPV-COOH}$) was from Lifetein (South Plainfield, NJ). Other chemicals and reagents, unless otherwise indicated, were obtained from Wako pure chemicals.

General Methods. ^1H NMR spectra were measured in CDCl_3 solution and referenced to TMS (0.00 ppm) using JEOL JNM-ECA 600 (600 MHz) spectrometers, unless otherwise noted. Peak multiplicities are used the following abbreviation: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. All reactions were performed under an air atmosphere unless otherwise noted, and tetrahydrofuran (THF) were purchased from Kanto Chemical Co., Inc., and other solvent was distilled. Unless otherwise noted, reagents were obtained from chemical sources and without further purification.

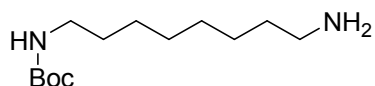
Recombinant expression and purification of anti-BGP scFv. *E. coli* SHuffle T7 Express lysY cells were transformed with pSQ(KTM219)¹ and cultured at 30 °C for 16 h in LBA medium (LB medium containing 100 $\mu\text{g}/\text{mL}$ ampicillin) and 1.5% agar. A single colony was picked and grown at 30 °C in 4 mL of LBA medium until OD_{600} of 0.9 was reached, from which 1.6 mL were used to inoculate 100 mL of LBA medium. The cells were cultured at 30 °C until an OD_{600} of 0.6 was reached, when 0.4 mM isopropylthio- β -galactopyranoside was added. The solution was incubated for 16 h at 16 °C and centrifuged ($8000 \times g$, 20 min, 4 °C). The pellet was resuspended in 10 mL of Talon wash buffer (50 mM phosphate, 0.3 M sodium chloride (NaCl), 5 mM imidazole, pH 7.4) and sonicated. After centrifugation ($8000 \times g$, 20 min, 4 °C), the supernatant was incubated with 0.2 mL of Talon metal affinity resin on a rotating wheel for 30 min at 25 °C. The beads were transferred to a Talon disposable gravity column, and washed three times with 8 mL Talon wash buffer. After adding 4 mL of Talon elution buffer (50 mM

phosphate, 0.3 M NaCl, 0.5 M imidazole, pH 7.4) and incubation at 25 °C for 30 min, the eluent was collected. The eluent was subjected to an ultrafiltration device and equilibrated with PBS (10 mM phosphate, 137 mM NaCl, 2.7 mM potassium chloride, pH 7.4) and concentrated to 250 μ L. The expression and purification of the protein were confirmed using a SDS-PAGE analysis, and the concentration of the protein was determined using ImageJ software (National Institutes of Health, Bethesda, MD) with the varied concentration of bovine serum albumin (BSA) as a standard.

Synthesis of IBA-C8-TAMRA



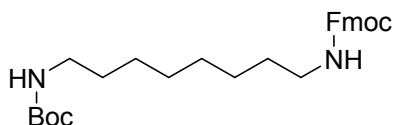
Compound S1



To a solution of 1,8-diaminooctane (**1**, 6.6 g, 45.8 mmol) in CHCl_3 (200 mL) was added dropwise a solution of Boc_2O (2.0 g, 9.2 mmol) in CHCl_3 (20 mL). The resulting solution was stirred at room temperature for 44 h and evaporated to give a residue, which was purified by silica gel column chromatography ($\text{MeOH}/\text{CHCl}_3 = 10$ to 30%) to afford 2.2 g (97%) of the title compound as colorless solids.

$^1\text{H-NMR}$ (CDCl_3 , 600 MHz) δ : 1.30-1.54 (m, 21H), 2.67 (t, $J = 6.6$ Hz, 2H), 3.10 (br, 2H), 4.74 (br, 1H)

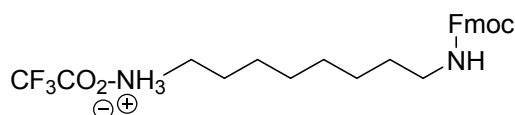
Compound S2



To a solution of Compound S1 (488 mg, 2.0 mmol) in CH_2Cl_2 (5 mL), cooled to 0 °C under argon, were added *N,N*-diisopropylethylamine (0.68 mL, 4.0 mmol) and Fmoc-Cl (517 mg, 2.0 mmol), and the mixture was stirred at room temperature for 20 h. After addition of 1M HCl (aq), the resulting mixture was extracted with CH_2Cl_2 and the organic layer was washed with H_2O and brine, dried over MgSO_4 , filtered and concentrated in vacuo to give a crude product, which was purified by recrystallization from toluene-hexane to afford 664 mg (70%) of the title compound as colorless solids.

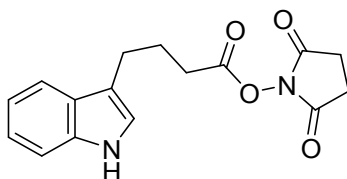
$^1\text{H-NMR}$ (CDCl_3 , 600 MHz) δ : 1.30 (s, 9H), 1.44-1.50 (m, 12H), 3.10 (m, 2H), 3.19 (q, $J = 6.6$ Hz, 2H), 4.22 (t, $J = 6.6$ Hz, 1H), 4.40 (d, $J = 7.2$ Hz, 2H), 4.50 (br, 1H), 4.74 (br, 1H), 7.31 (t, $J = 7.8$ Hz, 2H), 7.40 (t, $J = 7.8$ Hz, 2H), 7.60 (d, $J = 7.8$ Hz, 2H), 7.77 (d, $J = 7.8$ Hz, 2H)

Compound 2



To a solution of Compound S2 (186 mg, 0.40 mmol) in CH_2Cl_2 (10 mL) was added dropwise trifluoroacetic acid (3.3 mL, 43.4 mmol) and stirred at room temperature for 2 h. The resulting solution was evaporated to give a residue, which was precipitated by adding EtOAc-hexane (1:1) to afford 78 mg (54%) of the title compound as colorless solids. The resulting solids were used to the next reaction without further purification.

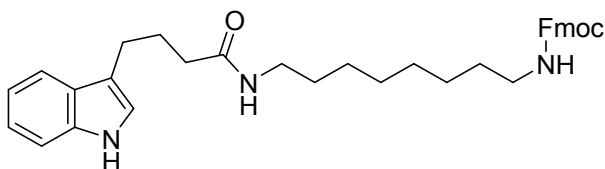
Compound 3



To a solution of 3-indolebutyric acid (406 mg, 2.0 mmol) in CH_2Cl_2 (20 mL) were added *N*-hydroxysuccinimide (345 mg, 3.0 mmol) and *N,N'*-diisopropylcarbodiimide (0.46 mL, 3.0 mmol), and the reaction mixture was stirred at room temperature for 20 h. After addition of H_2O , the resulting mixture was extracted with CH_2Cl_2 and the organic layer was washed with brine, dried over MgSO_4 , filtered and concentrated in vacuo to give a crude product, which was purified by silica gel column chromatography ($\text{MeOH}/\text{CHCl}_3 = 1\%$) to afford 543 mg (91%) of the title compound as brown oil.

$^1\text{H-NMR}$ (CDCl_3 , 400 MHz) δ : 2.15 (quintet, $J = 7.2$ Hz, 2H), 2.64 (t, $J = 7$ Hz, 2H), 2.82 (br, 4H), 2.90 (t, $J = 7$ Hz, 2H), 7.03 (s, 1H), 7.12 (t, $J = 8$ Hz, 1H), 7.19 (t, $J = 8$ Hz, 1H), 7.36 (d, $J = 8$ Hz, 1H), 7.61 (d, $J = 8$ Hz, 1H), 8.04 (brs, 1H); FAB-MS m/z 300 (M^+); HR FAB-MS m/z 300.1110 (M^+ , calcd for $\text{C}_{16}\text{H}_{16}\text{N}_2\text{O}_4$ 300.1110)

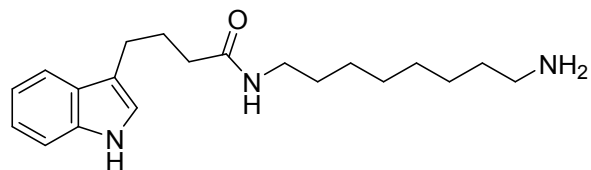
Compound 4



To a solution of Compound 3 (15 mg, 0.050 mmol) in CH_2Cl_2 (0.8 mL) were added Compound 2 (22 mg, 0.060 mmol) and *N,N*-diisopropylethylamine (0.013 mL, 0.075 mmol), and the reaction mixture was stirred at room temperature for 22 h. After addition of H_2O , the resulting mixture was extracted with CH_2Cl_2 and the organic layer was washed with brine, dried over MgSO_4 , filtered and concentrated in vacuo to give a crude product, which was purified by silica gel column chromatography ($\text{MeOH}/\text{CHCl}_3 = 1\%$) to afford 20 mg (73%) of the title compound as brown oil.

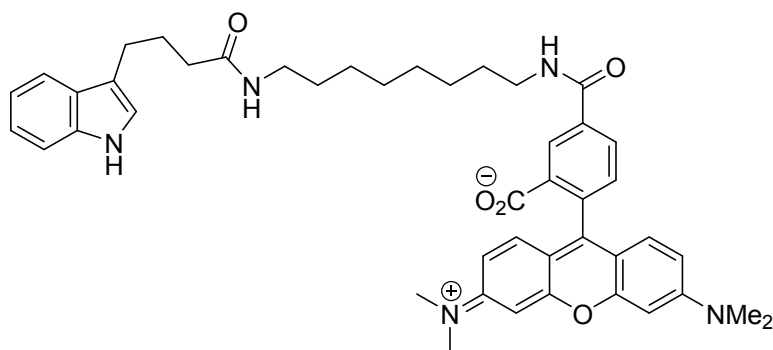
$^1\text{H-NMR}$ (CDCl_3 , 600 MHz) δ : 1.25-1.29 (m, 8H), 1.45-1.48 (m, 4H), 2.06 (quintet, $J = 7$ Hz, 2H), 2.22 (t, $J = 7$ Hz, 2H), 2.81 (t, $J = 7$ Hz, 2H), 3.17 (q, $J = 7$ Hz, 2H), 3.22 (q, $J = 7$ Hz, 2H), 4.22 (t, $J = 7$ Hz, 1H), 4.40 (d, $J = 7$ Hz, 4H), 4.74 (brs, 1H), 5.33 (brs, 1H), 6.99 (s, 1H), 7.11 (t, $J = 8$ Hz, 1H), 7.18 (t, $J = 8$ Hz, 1H), 7.31 (t, $J = 8$ Hz, 2H), 7.35 (d, $J = 8$ Hz, 1H), 7.40 (t, $J = 8$ Hz, 2H), 7.59 (m, 3H), 7.77 (d, $J = 8$ Hz, 2H), 8.03 (brs, 1H).

Compound 5



To a solution of Compound 4 (20 mg, 0.036 mmol) in DMF (0.8 mL) was added piperidine (0.018 ml, 0.18 mmol), and the mixture was stirred at room temperature for 40 min. The resulting solution was evaporated to give a crude product, which was used to next reaction without further purification.

Compound 6



To a solution of Compound 5 (2.8 mg, 9.4 μ mol) in CH_2Cl_2 (0.5 mL) was added TAMRA-NHS ester (2.5 mg, 4.7 μ mol) and stirred at room temperature for 24 h. The resulting solution was evaporated to give a crude product, which was purified by silica gel column chromatography (MeOH/ CHCl_3 = 5%) to afford 2.7 mg (84%) of the title compound as pink solids.

$^1\text{H-NMR}$ (CDCl_3 , 400 MHz) δ : 1.20-1.70 (m, 12H), 2.05 (quintet, $J = 7$ Hz, 2H), 2.23 (t, $J = 7$ Hz, 2H), 2.80 (t, $J = 7$ Hz, 2H), 3.00 (s, 12H), 3.24 (q, $J = 6$ Hz, 2H), 3.48 (q, $J = 6$ Hz, 2H), 5.47 (brs, 1H), 6.38-6.51 (m, 5H), 6.61 (d, $J = 9$ Hz, 2H), 6.99 (s, 1H), 7.09 (t, $J = 7$ Hz, 1H), 7.16 (t, $J = 7$ Hz, 1H), 7.23 (m, 1H), 7.34 (d, $J = 8$ Hz, 1H), 7.59 (d, $J = 8$ Hz, 1H), 8.16 (d, $J = 8$ Hz, 1H), 8.22 (brs, 1H), 8.28 (s, 1H); FAB-MS m/z 742 ($\text{M}^+\text{+H}$); HR FAB-MS m/z 742.3968 ($\text{M}^+\text{+H}$, calcd for $\text{C}_{45}\text{H}_{52}\text{N}_5\text{O}_5$ 762.3963)

Photocrosslinking of IBA-C8-TAMRA. 0.2 nmol of purified anti-BGP scFv or commercially available anti-HSA antibody was pre-incubated with 3 nmol of IBA-C8-TAMRA in a total volume of 10 μ L of PBS (pH 7.4) for 1 h prior to ultraviolet (UV) light exposure at room temperature. UV light (1 J/cm^2 , 254 nm) was irradiated to the sample using a shortwave crosslinker CL-1000 (UVP, Upland, CA). UV exposure was conducted on the top of PCR tube placed at a fixed distance of 9 cm from the UV light source.

Purification of Q-body. IBA-C8-TAMRA conjugated anti-BGP scFv type Q-body was purified using His-tag and Flag-tag as follows: The sample after UV exposure was subsequently mixed with 190 μ L of His wash buffer (20 mM phosphate, 0.5 M NaCl, 5 mM imidazole, pH 7.4) and incubated with 10 μ L of His Mag Sepharose Ni beads on a rotating wheel at room temperature for 30 min. The beads were washed three times with 1 mL of His wash buffer on a magnetic rack. After adding 500 μ L of His elution buffer (20 mM phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4) and incubating at 25 $^{\circ}$ C for 15 min, the eluent was collected on a magnetic rack and applied to Nanosep Centrifugal-3 k ultrafiltration. After equilibrating twice with 500 μ L of PBST by centrifuge ($14\,000 \times g$, 20 min, 4 $^{\circ}$ C), the supernatant was concentrated to 100 μ L and Anti-DYKDDDDK tag antibody magnetic beads (10 μ L) were added to the supernatant. After incubation at room temperature for 1 hour, the beads were washed three times with 1 mL of Flag wash buffer (20 mM phosphate, 0.5 M NaCl, 60 mM imidazole, 0.1% polyoxyethylene(23)lauryl ether, pH 7.4) on a magnetic rack and incubated with 130 μ L of wash buffer that contained 50 μ g of Flag peptide at room temperature. After 1 hour, the eluent was collected on a magnet, and applied to 7 k-desalting spin column for changing the buffer to PBST.

TAMRA-conjugated anti-HSA IgG type Q-body was purified using protein G magnetic beads as follows: The sample after UV exposure was subsequently mixed with 490 μ L of Tris-buffered saline (TBS: 50 mM Tris, 150 mM NaCl, pH 7.6) and incubated with 50 μ L of protein magnetic beads on a rotating wheel at room temperature for 1 hour. The beads were washed three times with 500 μ L of TBS on a magnetic rack. After adding 100 μ L of 0.1 M glycine, pH 2.0 and incubating at room temperature for 10 min, the eluent was collected on a magnetic rack and neutralized the low pH by adding 15 μ L of 1 M Tris, pH 8.5. Subsequently, the sample was applied to 7 k-desalting spin column for changing the buffer to PBST.

SDS-PAGE analysis. 5 μ L of Q-body was mixed with 5 μ L of SDS loading buffer (0.125 M Tris-HCl, 4% (w/v) SDS, 20% (w/v) glycerol, 0.01% (w/v) BPB, 100 mM DTT, pH 6.8), boiled at 95 $^{\circ}$ C for 5 min, and loaded to 12.5% PAGE. Precision Plus Protein Dual Color Standards (Bio-Rad, Hercules, CA, USA) were used as protein standards. After electrophoresis, a fluorescence image was obtained using a transilluminator with excitation at 500 nm (Gelmière, Wako). The protein concentration was determined after Coomassie Brilliant Blue or Silver staining by comparing with varied concentrations of BSA standard using ImageJ software. Also, the labeling yield was calculated by comparing the fluorescent band intensity with varied concentrations of rhodamine (TRITC)-conjugated IgG (Jackson Immuno research, West Grove,

PA, USA) using ImageJ software. The TAMRA concentration in this antibody was determined by comparing its fluorescence intensity with free TAMRA dye in 7 M guanidium hydrochloride (GdnHCl), 100 mM dithiothreitol (DTT).

Fluorescence measurements. 10 ng of Q-body in 250 μ L of PBST was poured in a 5 \times 5 mm² quartz cell (Starna Scientific, Hainault, UK), and various concentrations of BGP-C7 were added in titration at 3 min intervals, after each fluorescence spectrum was measured at 25°C using the fluorescence spectrophotometer Model FP-8500 (Jasco, Tokyo, Japan). To denature the protein, 7 M GdnHCl, 100 mM of DTT were added instead of PBST in a cell and the same procedure was performed. Both the excitation and emission slit widths were set to 5.0 nm. The excitation wavelength was 546 nm. Dose–response curves were made by fitting the intensities at the maximum emission wavelength of each Q-body using the Kaleida Graph 4.1 (Synergy Software, Reading, PA). The EC₅₀ and detection limit values were calculated from the curve fitting to a 4-parameter logistic equation.

Supporting references

SR1 H.J. Jeong, T. Kawamura, J. Dong and H. Ueda, *ACS Sens.*, 2016, **1**, 88–94.

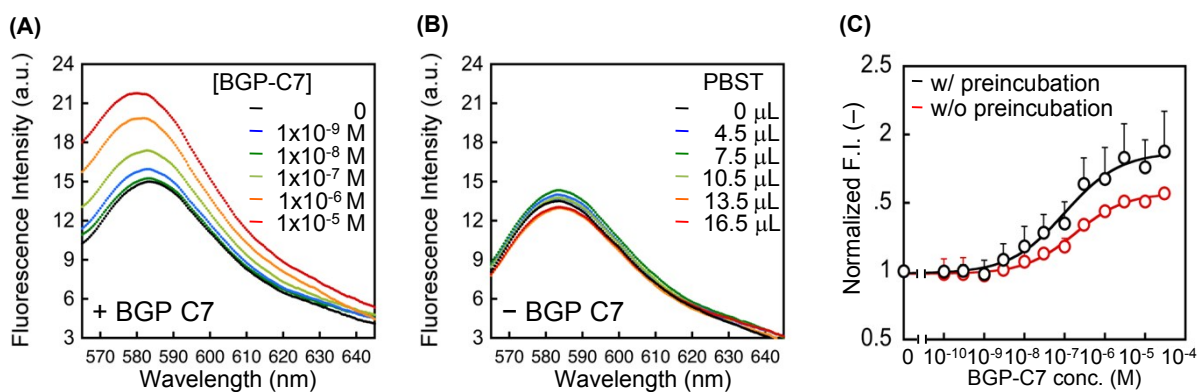


Fig. S1 Fluorescence spectra of anti-BGP Q-body with and without antigen, and the effect of pre-incubation. (A) The fluorescence spectra of the Q-body in the presence of BGP-C7 at the indicated concentrations. (B) The same as in (A) except that PBST buffer, the solvent of BGP-C7, was added. (C) A titration curve of the peak fluorescence intensity of the Q-body with or without pre-incubation prior to UV exposure. The normalised fluorescence intensity (F.I.) is the relative value with respect to that in the absence of antigen.

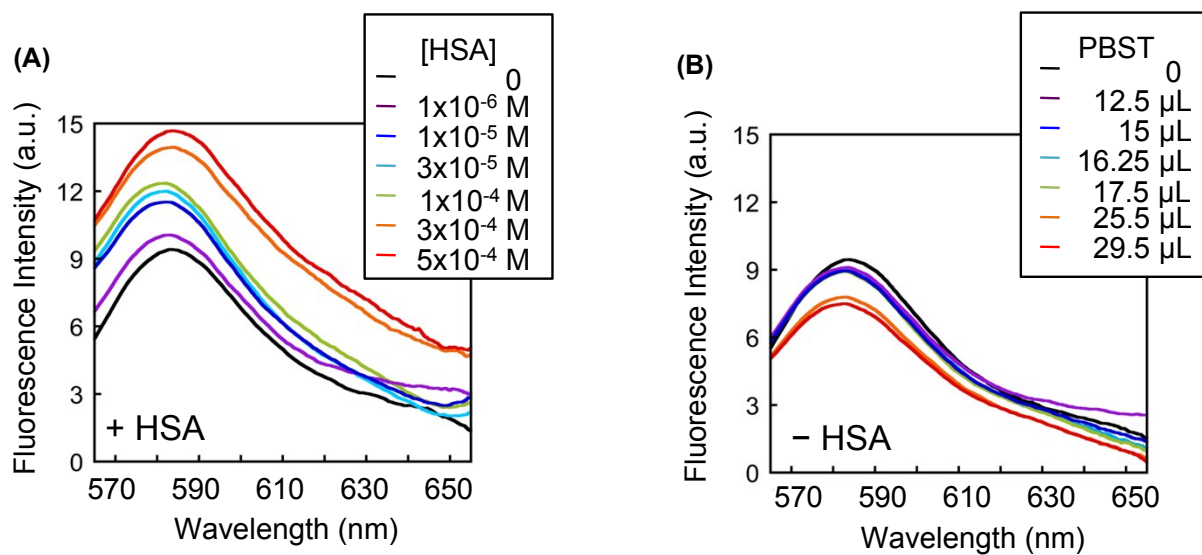


Fig. S2 (A) Fluorescence spectra of anti-HSA Q-body in the presence of HSA at the indicated concentrations. (B) The same as in (A) except that PBST, the solvent of HSA, was added.