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Electronic Supplementary Information (ESI)

Human Serum Albumin as Intrinsic Signal Amplification Amplifier for Ultrasensitive Assay of Prostate-Specific Antigen in Human Plasma

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EXPERIMENTAL SECTION

Chemicals and Apparatus. All oligonucleotides were purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). The oligonucleotides were purified by high-performance liquid chromatography (HPLC) and dissolved in highly pure water as stock solutions. HSA, GndHCl, IgG, hemoglobin, protease K, lysozyme, trypsin, thrombin, and insulin were purchased from Sigma-Aldrich. PSA was obtained from Keygen Biotech. Co. Ltd. (Nanjing, China). The common amino acids including glycine (Gly), serine (Ser), aspartic acid (Asp), glutamic acid (Glu), histidine (His), cysteine (Cys), tryptophane (Trp), and lysine (Lys) were purchased from Alfa Aesar (Tianjing, China). Other reagents and solvents were provided by Sinopharm Chemical Reagent Co., Ltd. (China). The PSA (Human) ELISA Kit was purchased from Fitzgerald (USA). The phosphate-buffered saline (PBS, pH 7.4) contained 137 mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, and 1.8 mM KH_2PO_4. Unless stated, all chemicals were used as prepared. Ultrapure water (18.2 MΩ cm) from a Milli-Q system with a Pyrogard filter (Millipore, MA, USA) was used for preparation of solution.

^{1}HNMR spectra were measured on a Bruker DRX-400 spectrometer operating at 400 MHz. The fluorescence spectra were carried on a PTI ASOC-10 Fluorescence System (Photo Technology International, Birmingham, NJ, USA). UV-Vis absorption spectra were recorded on a Shimadzu UV-2600 spectrophotometer (Kyoto, Japan). The circular dichroism (CD) spectra were performed on a MOS-500

spectropolarimeter (Biologic, France). Transmission electron microscope (TEM) images of AuNPs were performed on a Tecnai F20 with an accelerating voltage of 200 kV. The hydrodynamic diameters were recorded by the Malvern ZetaSizer Nano instrument (Malvern, Worcestershire, UK). The fluorescence of samples in 96-well microtiter plates was read using a multi-functional plate reader Infinite M1000 (Tecan, Switzerland). Fluorescence imaging of 96-well plates were obtained by an IVIS Lumina II in vivo imaging system (Caliper Life Science, USA).

Synthesis of EBCB. EBCB was synthesized by a modification of the procedure reported by Li et al. as outlined in Scheme S1.[1]

Ethyl-4-(3,6-dibromo-9H-carbazol-9-yl) butanoate (Compound 1). Typically, 3, 6-dibromocarbazole (1.30 g, 2 mmol), ethyl-4-bromobutanoate (2.30 mL, 8 mmol) was added in a DMF solution (80 mL) contained KOH (2.24 g, 20 mmol) and KI (160 mg, 0.48 mmol). The mixture was stirred for 10 h at 60 ^{o}C under the protection of argon atmosphere. Subsequently, 200 mL H_2O was added and the final mixture was extracted with ethyl acetate. The organic layer was washed twice with water and brine. After the anhydrous Na_2SO_4 drying, filtration, decompress concentration, and chromatography separation, the final white powder compound 1 was obtained.

Ethyl-4-[3, 6-Bis(4-vinylpyridium iodine)-9H-carbazol-9-yl)] butanoate (Compound 2). In a high pressure bottle, Compound 1 (1.5 g, 1.7 mmol), tri-o-tolyl phosphine (200 mg), palladium(II) acetate (10 mg) and 4-vinylpyridine (2.6 mg) were added into the triethylamine (6 mL)/acetonitrile (18 mL) solution. After the reaction at 105 ^{o}C for 48 h, the mixture was transferred to a flask and the solvent was removed under

reduced pressure to give a yellow crude product, which was purified by chromatography on silica gel using CH_2Cl_2/CH_3OH (5:1, V/V) as an eluent to give compound 2 as earth yellow solid.

EBCB (Compound 3). Excess CH_3I and compound 2 (0.9 g) in acetonitrile/DMF was then refluxed for 4h, the orange red powder, compound 3 was obtained with a 87% yield after recrystallization twice using methanol. ^{1}HNMR (400 MHz, DMSO) δ(ppm) 8.83 (d, *J* =4.8 Hz, 4H), 8.63 (s, 2H), 8.23(t, *J* =16.4 Hz, 4H), 7.97 (d, *J* =8.8 Hz, 2H), 7,82 (d, *J* =9.2 Hz, 2H), 7.58 (d, *J* =16.4 Hz, 2H), 4.52(t, *J* =12.8 Hz, 2H), 4.26(s, 3H), 4.24 (s, 3H), 3.97 (m, 2H), 2.41(t, *J* =14.0 Hz, 2H), 2.07 (t, *J* =13.6 Hz, 2H), 1.12(t, *J* =14.0 Hz, 3H).

Investigation of Binding Site between EBCB and HSA. Stock solutions of HSA and EBCB (250 μM) were prepared with PBS. For a typical detection, EBCB solution (4 μL) and HSA solution (50 μL) at different concentrations were added in PBS buffer to a final volume of 200 μL. The corresponding fluorescence was measured with 485 nm excitation, and the emission spectrum was recorded from 500 to 750 nm.

The binding site of EBCB to HSA was investigated by the competitive binding assay. HSA (0~10 mg/mL) was premixed with EBCB for the collection of the fluorescence spectrum. Then, solutions of dansylamide (DNSA) or ibuprofen dissolved DMSO with different amounts was added in the mixed solution, respectively. The resultant mixtures were subjected for the measurement of fluorescence.

Molecular Docking. Molecular docking was carried out using software AutoDock

Vina.[2] The X-ray structure of HSA (PDB ID: 3B9M[3]) was retrieved from the Protein Data Bank (http://www.rcsb.org/pdb) for docking calculation. Water molecules and ligands were removed. To prepare for both the protein and small molecule, all hydrogens were added firstly, Gasteiger charges were computed, and non-polar hydrogens were merged. A definition of the active site is provided as the ligand binding site I, center coordinates of the grid box were 32.01, -0.28, 9.51 (x, y, z). The sizes of the x, y, z axes are 30 × 30 × 30. The protein was considered rigid for the docking study. All allowed torsional bonds of the ligand were considered rotatable. Default values in the software were set for other parameters. The conformation of the docked ligand to the protein was selected according to the predicted binding free energy. Protein-ligand interactions figures were generated using PyMOL.

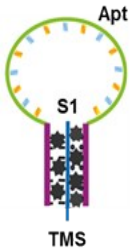
Fabrication of TMS-Au Nanoprobe. Au nanoparticles (AuNPs) were prepared by a sodium citrate reduction method of $HAuCl_4$ and modified with TMS on the basis of the reported methods.[4] The TMS consists of a PSA-specific aptamer sequence (Apt) flanked by two arm segments and a triplex-forming intermediate oligonucleotide (S1). Briefly, the S1 probe was incubated with the solution containing AuNPs for 12 h. Then, 2 M sodium chloride solution was added to the mixture solution drop by drop at every 6 h period while the final concentration was achieved to 0.3 M. The solution was centrifuged for 30 min (13000 g) and resuspended in 0.01 M phosphate buffer saline three times. Apt was added to the PBS buffer to form TMS-modified AuNPs. To acquire the mean number of TMS on one single particle, we divided the conjugated TMS concentration by the initial concentration of AuNPs. The

concentration of TMS on the AuNPs surface was measured by fluorescence experiment with fluorophore-labeled Apt. The supernatant's maximal fluorescence peak, with free Apt isolated from the particles, was transformed to molar concentrations of the fluorophore modifying on oligonucleotide by comparison to a standard linear calibration curve. Standard curves were measured with given concentrations of fluorophore-labeled Apt DNA using uniform buffer pH and salt concentrations. Finally, 10 μL of 500 μM EBCB were added to the mixture to form TMS-Au detective probe for PSA. The nanoparticles were centrifuged, resuspended and stored at 4 °C.

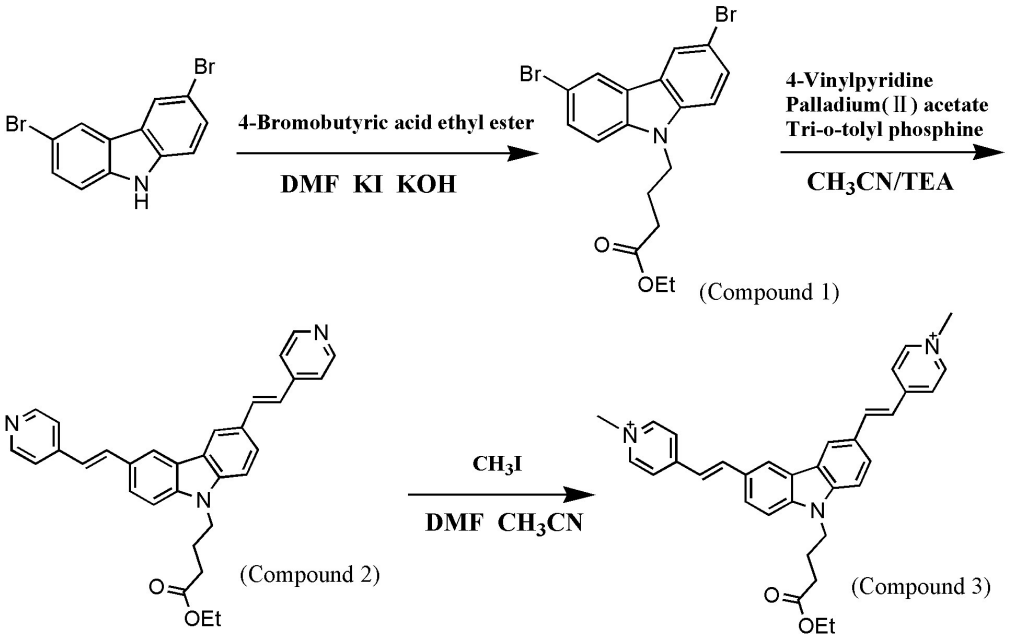
Fluorescence Measurements of PSA in PBS. In a quartz cell contained 1.0 nM TMS-Au nanoprobe probe, following the additions of PSA in a certain concentration range, the solutions were incubated at room temperature. After 10 min shake at room temperature, 42 mg/mL HSA was added to each well to amplify the detectable fluorescent signal. Then the assay plate was shaken at room temperature for 5 min. The fluorescence of each well at 450 nm was read using a microplate reader.

Assays of Clinical Serum Samples. To assess the clinical applicability of the developed self-served fluorescence amplification, we performed the assays on clinical serum samples. Human blood samples were obtained from a healthy female donor (negative) and a patient suffering from prostate cancer (positive). After blood was spun down (8000 rpm, 10 min) to remove the red blood cells, 1.0 nM TMS-Au nanoprobe and 10 μM DNSA were introduced and their fluorescence intensities were monitored by a microplate reader.

Table S1. Oligonucleotides used in this work.



Entry	Sequence (5'-3')
S1	AAAAGAAGAAGGGGGT-SH
Apt1	TTCTTTTATTAAAGCTCGCCATCAAATAGCTTTTCTT
Apt2	CTTCTTTTATTAAAGCTCGCCATCAAATAGCTTTTCTTC
Apt3	TCTTCTTTTATTAAAGCTCGCCATCAAATAGCTTTTCTTCT
Apt4	TTCTTCTTTTATTAAAGCTCGCCATCAAATAGCTTTTCTTCTT



Scheme S1. The synthetic routes of EBCB.

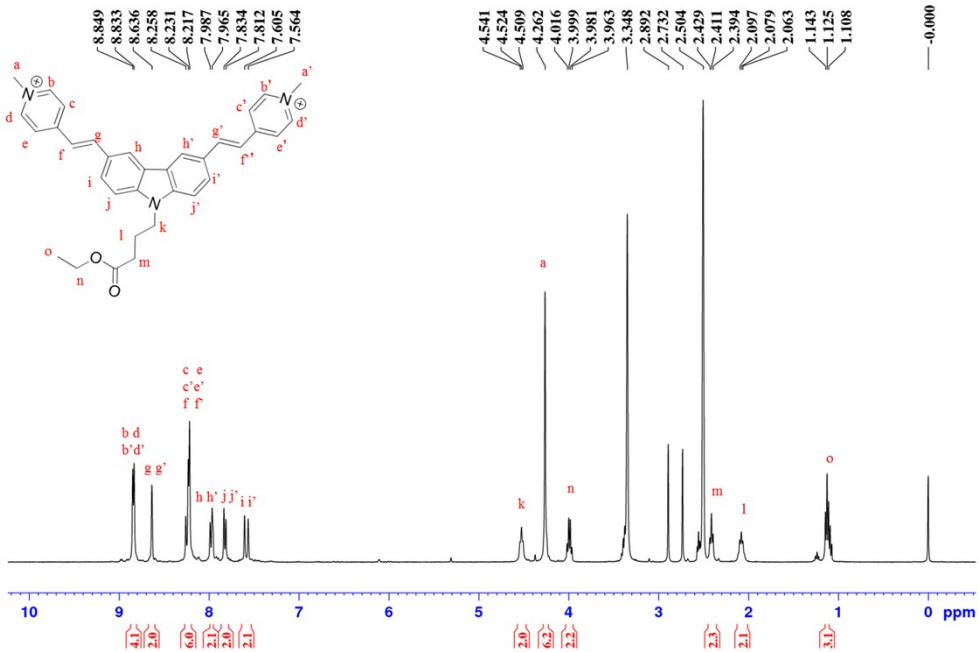


Figure S1. ^{1}HNMR of EBCB.

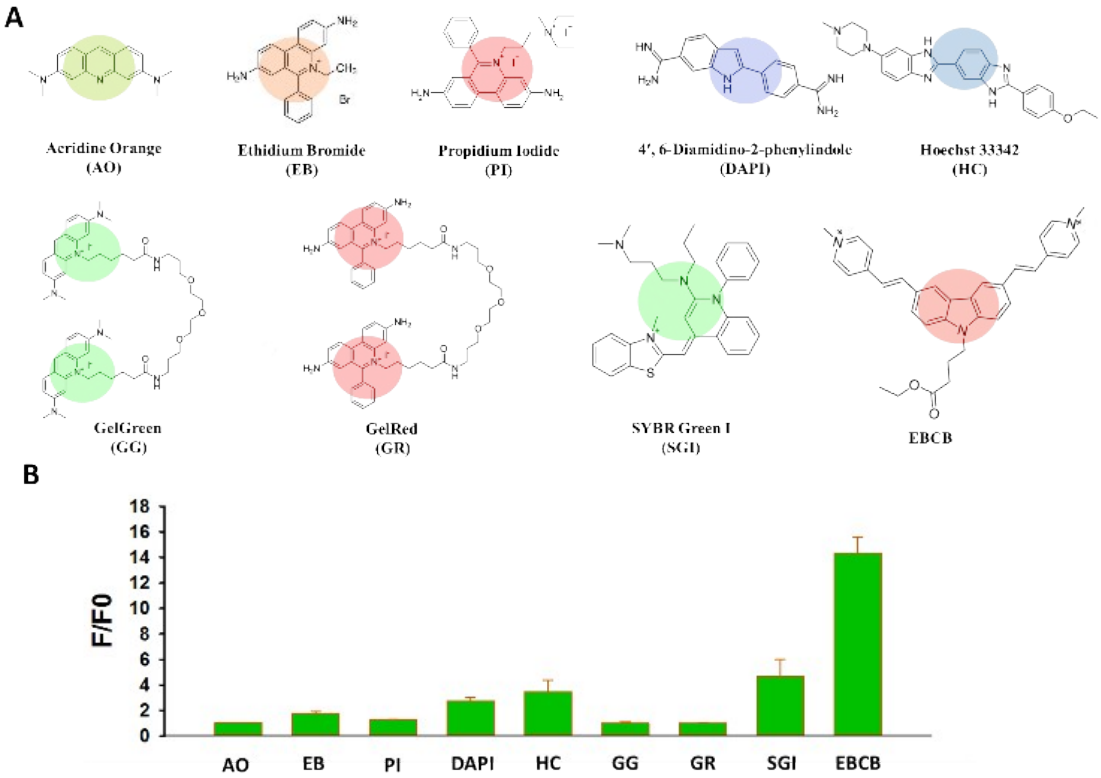


Figure S2. (A) Molecular structures of dye candidates for screening HSA-enhanced fluorophore. (B) Fold of fluorescence enhancement of dye candidates by HSA. Here

F0 and F are the fluorescence intensity of each dye (1.0 μM) in 10 mM phosphate buffer in the absence and the presence of 20 mg/mL HSA, respectively. The fluorescence of the dyes was recorded at their maximal excitation/emission wavelengths, respectively.

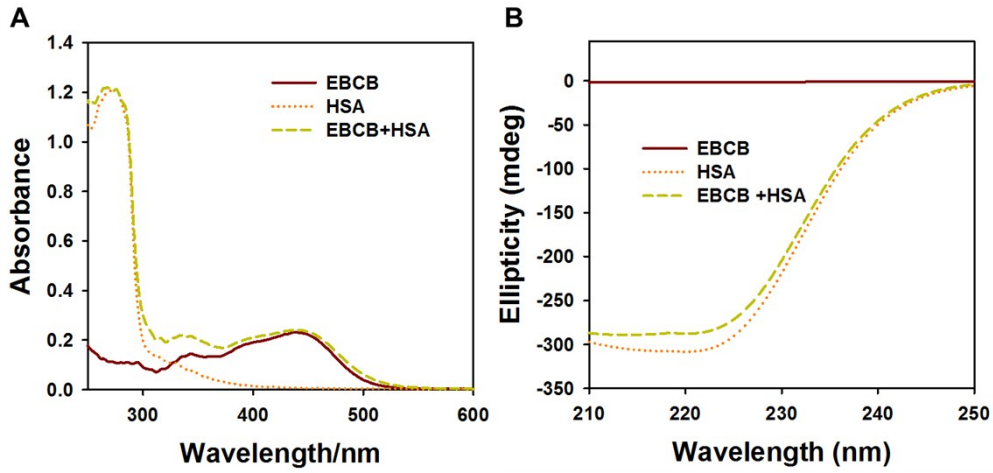


Figure S3. UV-visible absorption spectra (A) and circular dichroism spectra (B) of EBCB, HSA and EBCB-HSA complex.

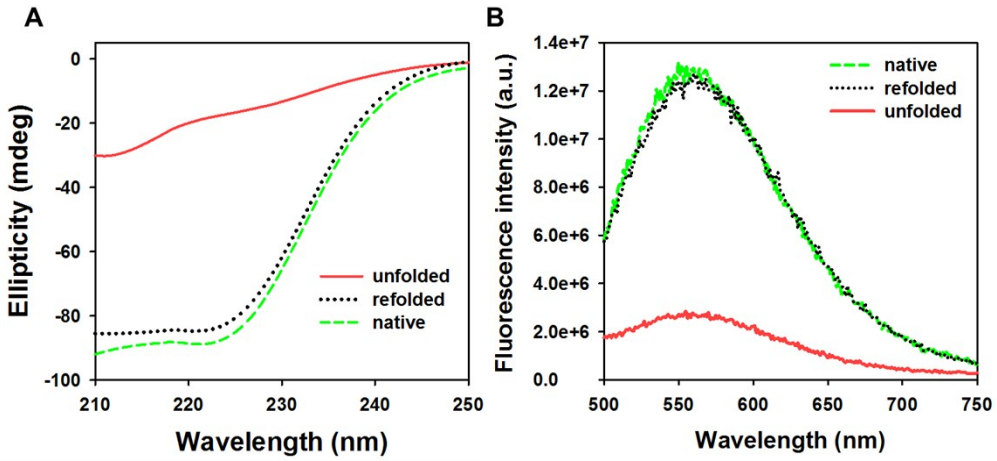


Figure S4. (A) CD spectra of EBCB bound with refolded HSA in the PBS buffer and unfolded HSA in the 6 M GndHCl solution. (B) Fluorescence spectra of refolded

HSA in the PBS buffer and unfolded HSA in the 6 M GndHCl solution. The spectra for the native HSA are given for comparison.

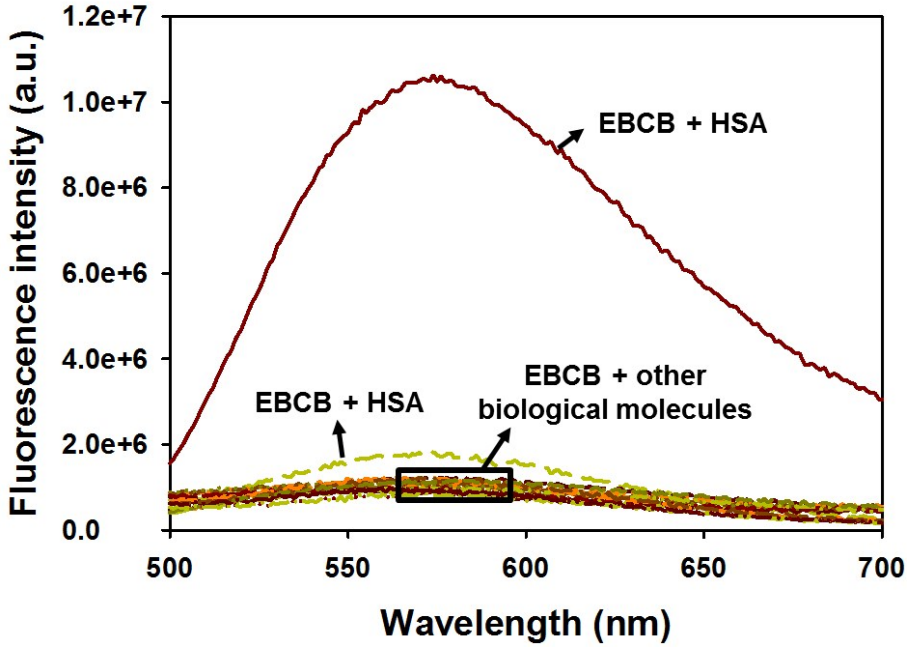


Figure S5. Fluorescence emission spectra of EBCB upon titrating with various biological molecules in 10 mM PBS (pH 7.4). [HSA] = 20 mg/mL, [other additives] = 0.6 mM.

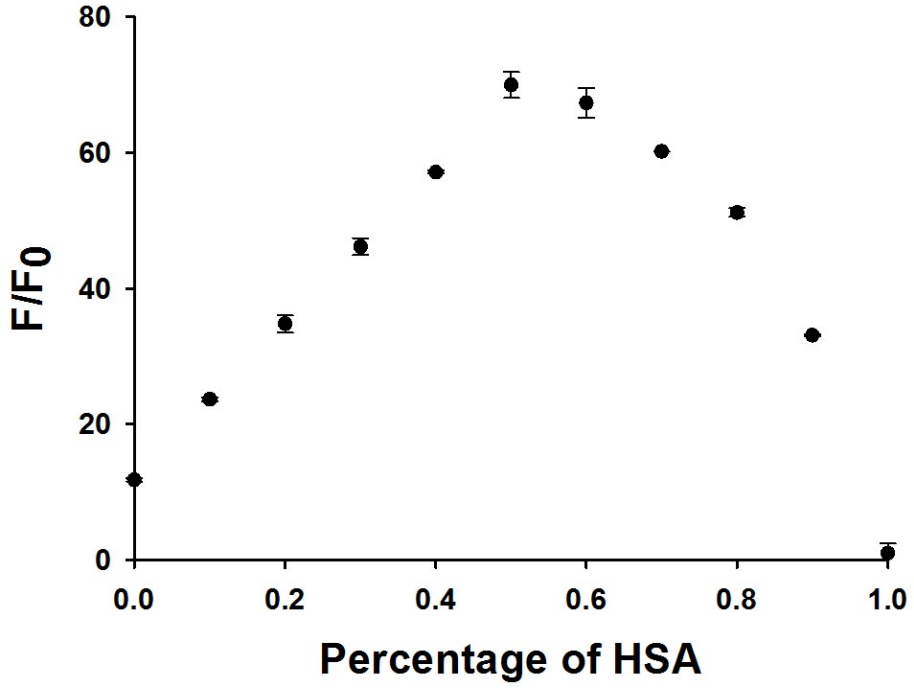


Figure S6. Determination of the binding stoichiometry of EBCB to HSA. The total concentration of EBCB and HSA is 50 μM.

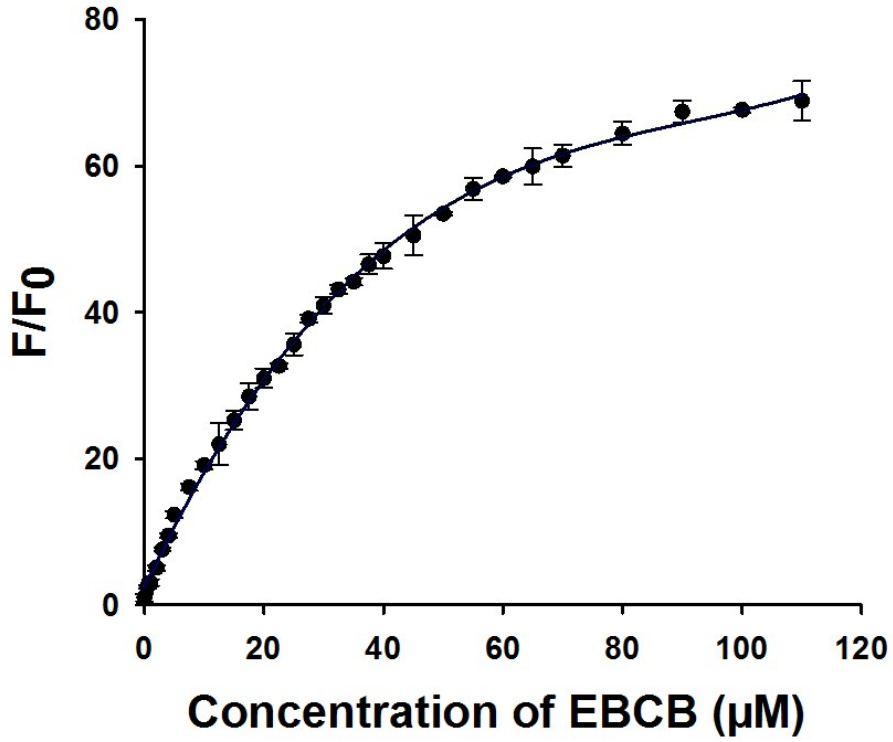


Figure S7. The titration curve of EBCB to HSA (40 mg/mL).

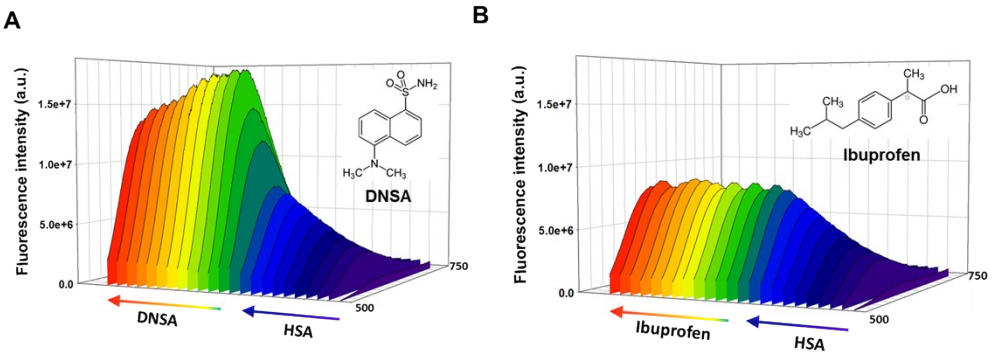


Figure S8. Fluorescence spectra of EBCB (10 μM) with the addition of different concentration of HSA (0 ~ 10 mg/mL), followed by adding (A) DNSA (0 ~ 220 μM), and (B) ibuprofen (0 ~ 220 μM). λ_{ex} =485 nm.

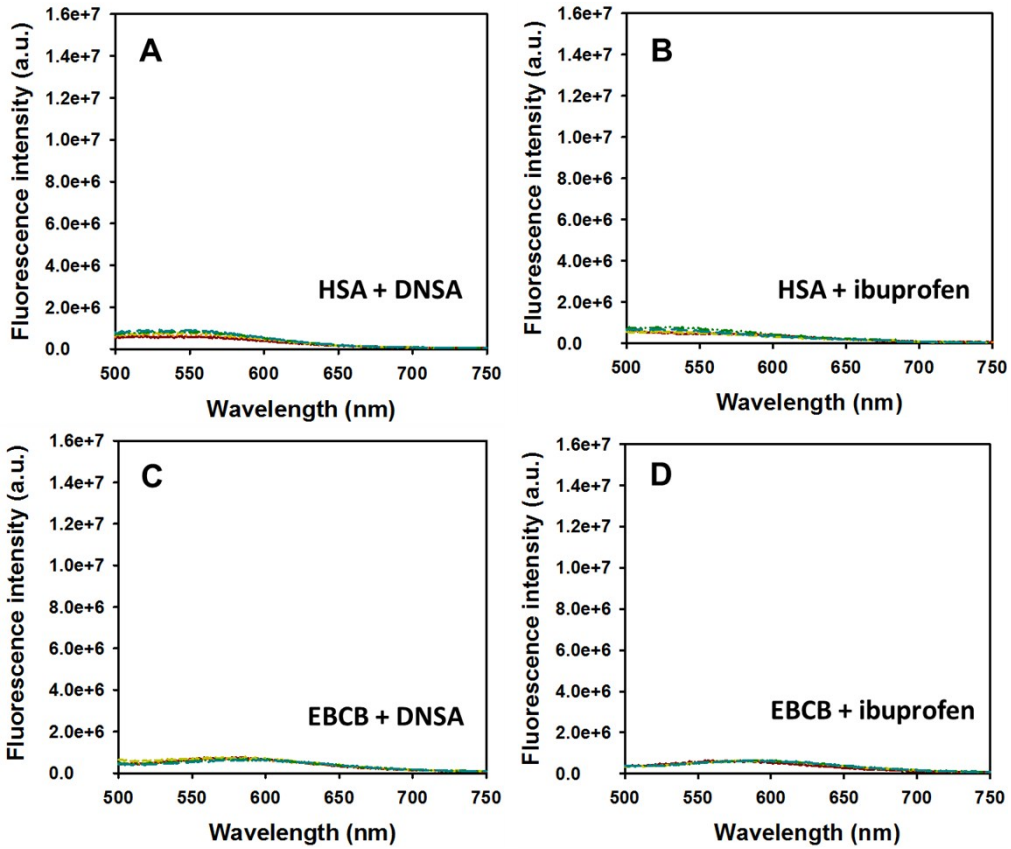


Figure S9. Effects of DNSA and ibuprofen on the fluorescent emission properties of EBCB and HSA at 575 nm, λ_{ex} = 485 nm.

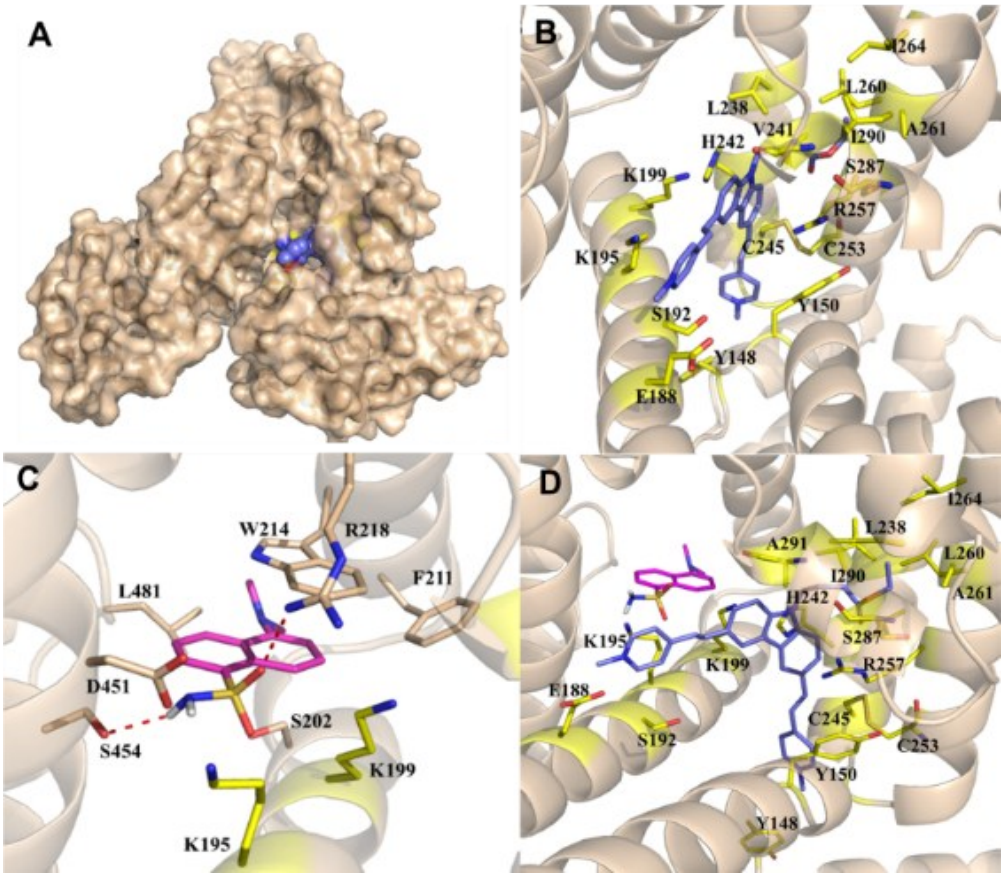


Figure S10. Minimum-energy conformation of binding of EBCB/DNSA inside the sudlow site I of HSA as suggested by the molecular docking study. (A) Overview of the docked conformation of EBCB to HSA; (B) A close view of the amino acid residues in the immediate vicinity of EBCB; (C) A close view of the amino acid residues in the immediate vicinity of DNSA; (D) Binding interactions made by DNSA and EBCB in HSA.

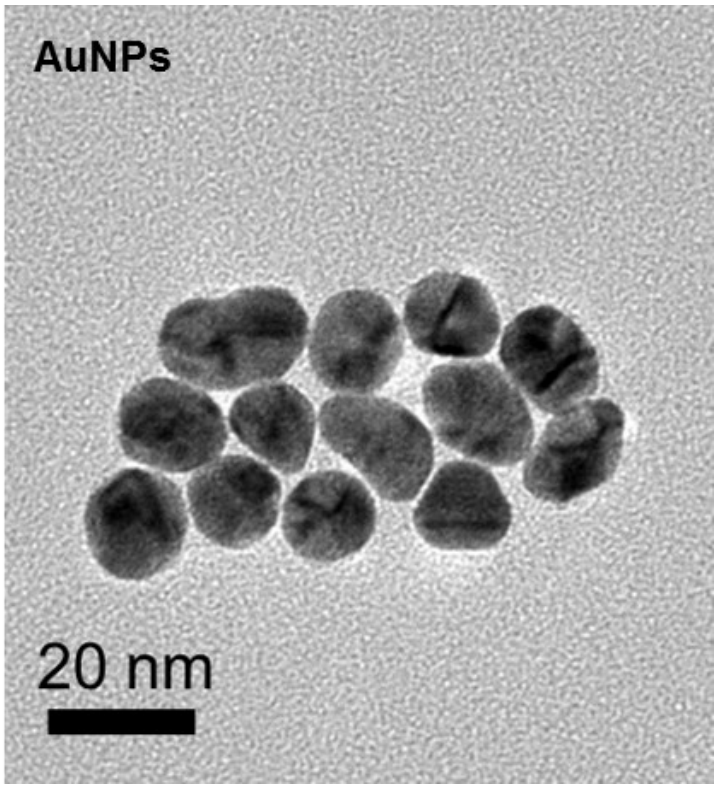


Figure S11. TEM images of 13-nm gold nanoparticles.

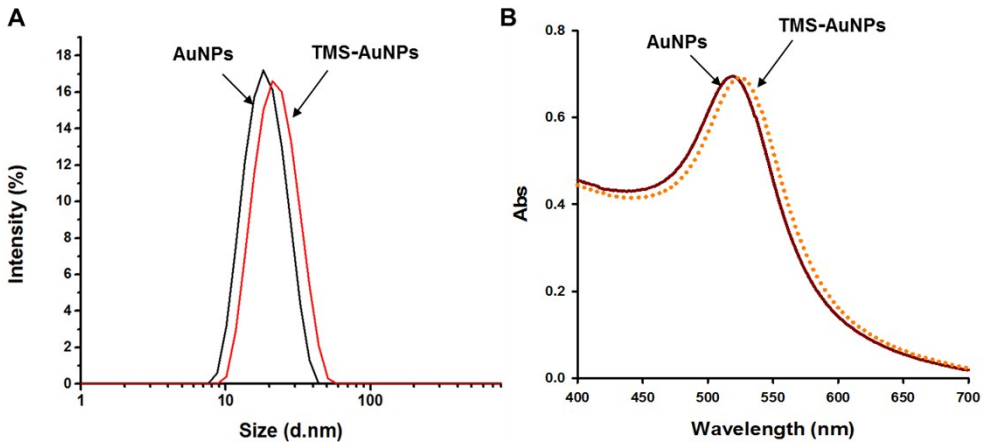


Figure S12. The hydrodynamic diameters (A) and UV-visible absorption spectra (B) of AuNPs and TMS-AuNPs.

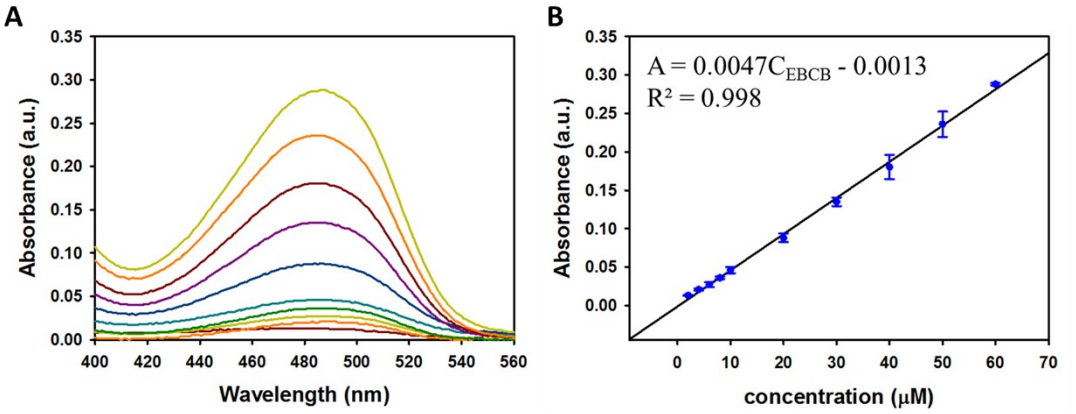


Figure S13. UV-Vis spectra (A) and calibration curve (B) upon the addition of known amounts of EBCB

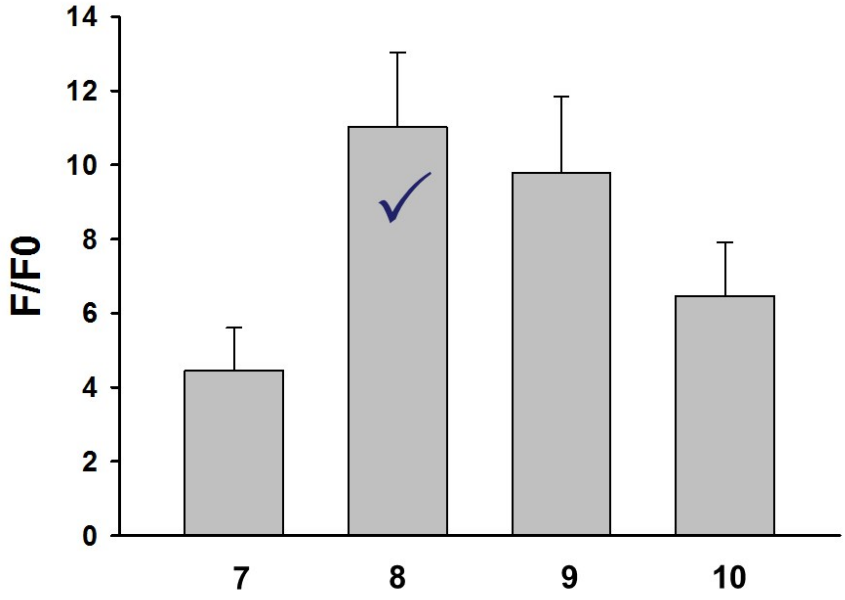


Figure S14. Effect of the triplex-stem length of nano-TMS probe on the PSA detection in PBS buffer contained 40 mg/mL HSA and 10 μM DNSA. The concentration of PSA was 1 ng/mL.

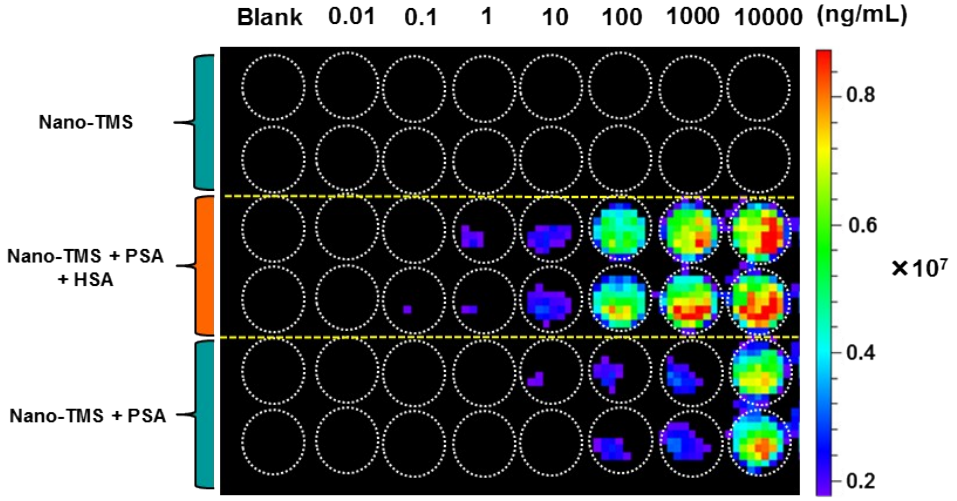


Figure S15. Fluorescence images of the proposed nano-TMS sensing system upon the addition of PSA in PBS buffer contained different agents in a 96-well PS plate.

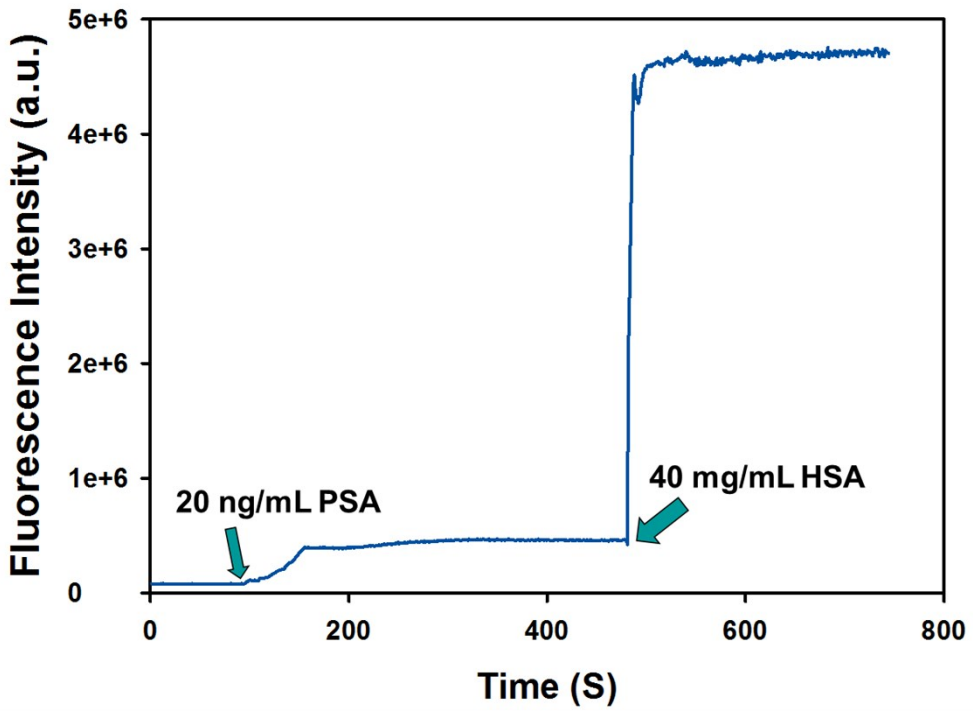


Figure S16. Real-time monitoring of fluorescence emission upon addition of 20 ng/mL PSA and 40mg/mL HSA in PBS buffer containing 10 μM DNSA and nano-TMS probe.

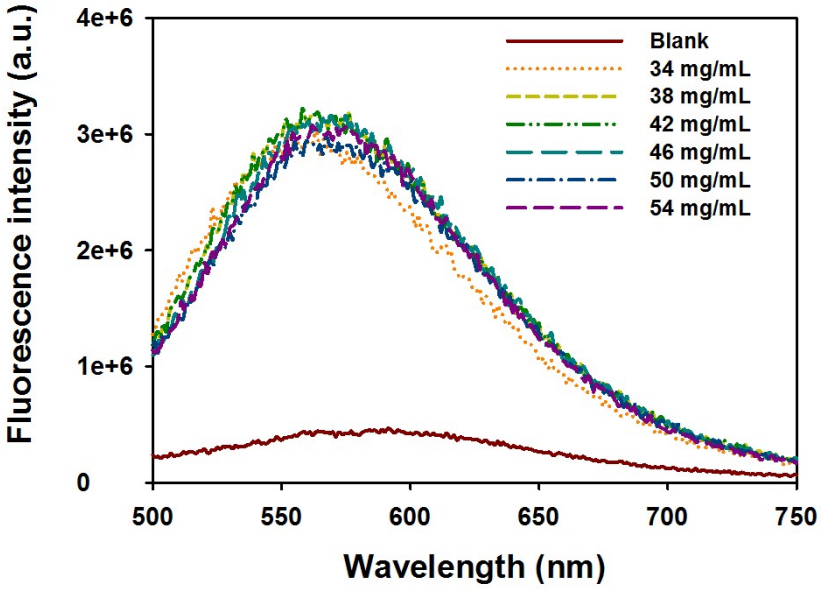


Figure S17. The assay of PSA in different concentrations of HSA in the range of 34 to 54 mg/mL. The concentration of PSA was 0.2 ng/mL.

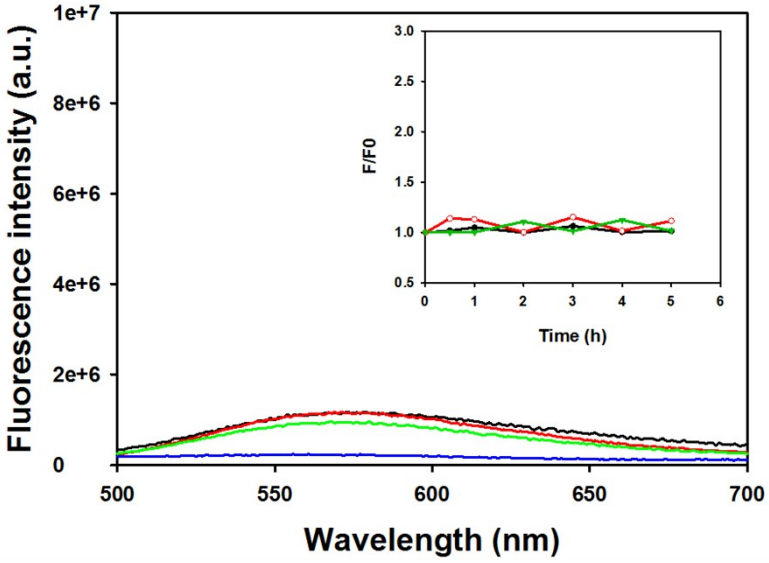


Figure S18. Fluorescence spectra of nano-TMS in the presence (black curve) or absence of DNase I (grass green curve) in buffer. The red curve represents the fluorescence spectra of nano-TMS in serum. The blue curve represents the blank serum. Inset: the corresponding curve by plotting F/F0 at 570 nm during a function of time.

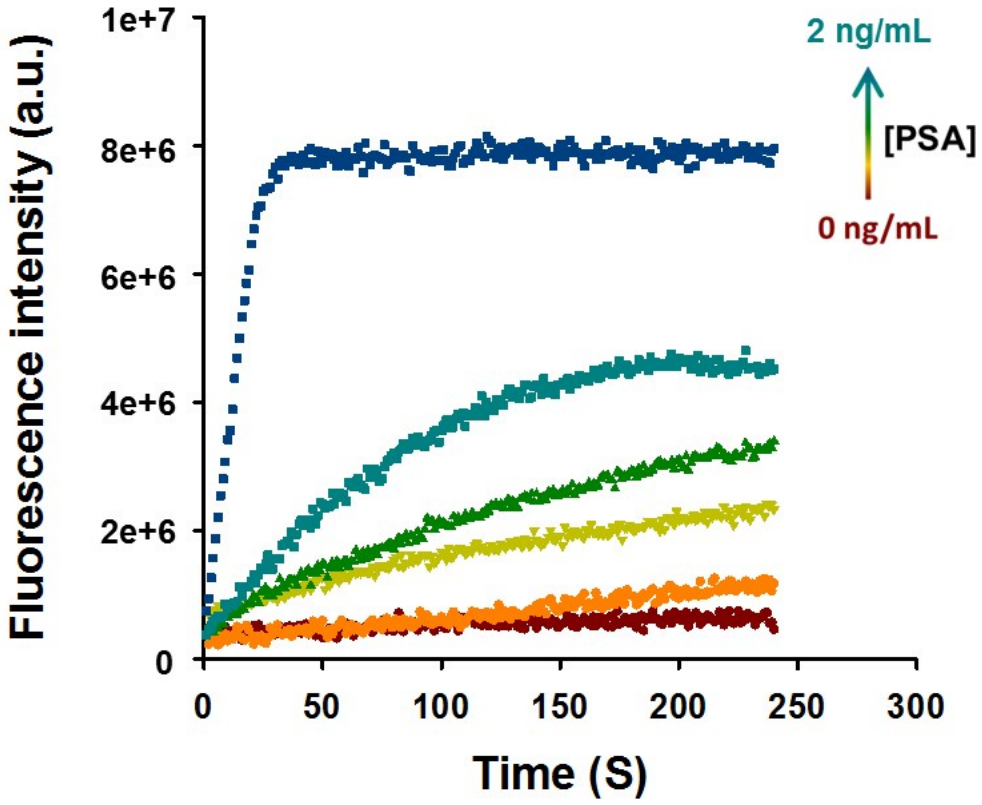


Figure S19. Real-time monitoring of fluorescence emission versus the concentration of PSA in serum containing 10 μM DNSA. The concentration of PSA was 0, 0.025, 0.05, 0.1, 0.5, 1.0, 2.0 ng/mL.

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