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Supporting Information

A microenvironment-sensitive red emissive probe with a large stokes shift for the specific recognition and quantification of serum albumin in complex biofluids and live cells

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Instrumentation:

The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 300 and 400 MHz spectrophotometer using tetramethylsilane as an internal standard in DMSO-d_6. The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Coupling constants, *J* were expressed in Hertz unit (Hz). The ESI-MS^{+} (m/z) spectra of the probe were measured on a HRMS spectrophotometer (model: QTOF Micro YA263). The Fourier transform infrared spectrum (4000–400 cm^{-1}) of the probe was obtained on a Perkin-Elmer RX I FT-IR spectrophotometer with a solid KBr disc. The UV-Vis absorbance spectral studies were carried out on an Agilent diode-array Spectrophotometer (Agilent 8453) using a 1 cm path length quartz cuvette in the wavelength range of 190-900 nm. Steady-state fluorescence spectroscopic measurements were performed on a PTI spectrofluorimeter (Model QM-40) by using a fluorescence free quartz cuvette of 1 cm path length. The excitation and emission slit widths were fixed at 3 nm. Fluorescence lifetimes were determined from time-resolved intensity decay by the method of time correlated single photon counting (TCSPC) measurements using a picosecond diode laser (IBH Nanoled-07) in an IBH fluorocube apparatus. The fluorescence decay data were collected on a Hamamatsu MCP photomultiplier (R3809) and examined by the IBH DAS6 software.

UV–Vis and fluorescence spectroscopic studies:

Stock solution of DCI-MIN (1×10^{-3} M) in DMSO was diluted to 2 μM for various spectroscopic studies by placing only 4 μL of the stock solution of DCI-MIN into PBS solution (pH 7.4) to a final volume of 2 mL. In the fluorescence selectivity experiment, the test samples were prepared by adding the appropriate amounts of the stock solutions of the respective proteins, enzymes, cations, anions and other bioanalytes into 2 mL of probe DCI-MIN solution (2 μM). For the fluorescence-titration experiments, another set of HSA standard solution (1×10^{-4} M) was prepared by diluting the earlier prepared 25 mg/mL stock solution in PBS medium and was gradually added into the DCI-MIN solution (2 μM). For the fluorescence experiments, excitation wavelength was set at 476 nm and emission was recorded from 490 to 780 nm. For the UV-vis studies the probe concentration was also fixed at 2 μM and the spectra were collected with proper background correction. For the fluorescence displacement studies, stock solutions of hemin, warfarin, and diflunisal (1 mM for each) were prepared in DMSO, deionized water, and DMSO, respectively. In this assay, the HSA solution premixed with DCI-MIN at a molar ratio of 1:4 was subsequently spiked with different amounts of hemin/warfarin/diflunisal and the resultant ternary mixtures were subjected to fluorescence measurement.

Steady-state Fluorescence Anisotropy:

Fluorescence anisotropy (*r*) measurements were carried out by considering the following equation described by Larsson *et al*.[S1]

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \qquad \text{(Eq. S1)}$$

Where, the polarizer positions were set at (0°, 0°), (0°, 90°), (90°, 0°), and (90°, 90°) to get I_{VV}, I_{VH}, I_{HV}, I_{HH} for excitation and emission signals respectively. G factor is defined as

$$G = \frac{I_{HV}}{I_{HH}} \qquad \text{(Eq. S2)}$$

Where, I_{HV} and I_{HH} are respectively the vertical and horizontal component of emission polarizer, keeping the excitation polarizer horizontal. G depends on slit widths and monochromator wavelength. The excitation and emission wavelengths were fixed at 476 and 602 nm respectively.

Fluorescence Lifetime Studies:

The TCSPC measurements were carried out in 10 mM PBS buffer solution of pH 7.4 for the fluorescence decay of DCI-MIN in the absence and presence of increasing concentration of HSA at 25 °C. During the TCSPC measurements the photoexcitation was fixed at 480 nm. The fluorescence decay curves were fitted to a biexponential function:

$$I(t) = A + \alpha_1 . e^{(-t/\tau_1)} + \alpha_2 . e^{(-t/\tau_2)} \qquad \text{(Eq. S3)}$$

Where, α_i represents the ith pre-exponential factor and τ_i denotes the decay time of component i (here i = 1, 2). The average lifetimes (τ_{avg}) for the fluorescence decay profiles were calculated by using the following equation:[S2]

$$\tau_{avg} = \sum_{i=1}^{2} \alpha_i . \tau_i / \alpha_i \qquad (Eq.S4)$$

Fluorescence quantum yield measurements:

Fluorescence quantum yields were calculated by adopting the reported strategy[S3] where relative measurement was carried out using Rhodamine 6G as reference ($\Phi_s = 0.94$ in ethanol) and by considering the following equation:

$$\Phi_u = \frac{A_s F_u \eta_u^2}{A_u F_s \eta_s^2} \times \Phi_s \qquad \text{(Eq. S5)}$$

Where, "Φ" is the quantum yield; "A" is the optical density; "F" is the measured integrated emission intensity; and "η" is the refractive index. The subscript "u" refers to the unknown sample, and subscript "s" refers to the standard reference with a known quantum yield.

Detection Limit:

The detection limit was calculated on the basis of the fluorescence titration with HSA. The fluorescence emission spectrum of DCI-MIN was measured 10 times to calculate the standard deviation of blank measurement. Then, the fluorescence emission at 602 nm was plotted as a function of the concentration of HSA from the corresponding titration experiment to evaluate the slope. The detection limit was then calculated using the following equation:[S4]

Detection limit = 3σ/k (Eq. S6)

Where "σ" is the standard deviation of blank measurement, and "k" is the slope between the fluorescence emission intensity versus [HSA].

Molecular Docking Study:

To determine the plausible binding sites within HSA and the mode of binding of DCI-MIN with HSA, molecular docking studies was carried out using docking program AutoDock (version 4.2). The X-ray crystal structures of HSA (PDB ID: 2BXE and 1O9X) were taken from RCSB Protein Data Bank for docking studies. To draw the structure of DCI-MIN, Chem3D Ultra 19.1 was used and further modification was carried out by using Gaussian 09W and AutoDock 4.2 programs. Gasteiger charges and polar hydrogen atoms were added to the protein and probe. Using the AutoGrid tool, a grid box with dimensions of 120 Å × 120 Å × 120 Å and 0.403 Å grid spacing were selected to accommodate the protein. The default values shown by the AutoDock program were used for other sets of parameters. The grid maps for energy were computed using AutoGrid, and docking calculations were carried out using the Lamarckian genetic algorithm (LGA).[S5,S6] The best optimized docked model with lowest binding energy was chosen for further analysis of docking studies and the output was best viewed using Discovery Studio.

Cell Cytotoxicity assay:

The cell cytotoxicity study of DCI-MIN was performed by MTT assay. At first, A549 cells were cultured in 24-well plates in Dulbecco's modified Eagle's (DMEM) medium (supplemented with 10% FBS and 1% penicillin−streptomycin) at 37 °C and under 5% CO_2. A549 cells were treated with different doses of DCI-MIN for 24 h and replenishing with fresh media. Then, 50 µL of an aqueous solution of MTT (5 mg/mL) was added to each well and incubated for 4 h. The produced purple formazan was dissolved in a DMF−water (1:1) solution mixture of sodium dodecyl sulfate, and the absorbance of the solution was measured at 570 nm in a microplate

reader. The relative cell viability was measured by assuming 100% cell viability for cells without any DCI-MIN.

Cell Imaging Study:

A549 cells were cultured over the glass cover slip in 24-well plate in DMEM (with 10% FBS + 1% penicillin−streptomycin) at 37 °C and 5% CO_2 atmosphere. After overnight growth, the cells were starved for adding DMEM without 10% FBS for 6 h, for live cell imaging of endogenous HSA. Then A549 cells were incubated with 2 μM DCI-MIN for 30 min and LysoTracker dye for 10 min in serum free DMEM media. The cells were washed with PBS buffer (pH 7.4) for three times to remove the residual molecules. Then fresh DMEM media was added to the washed cells and imaged under fluorescence microscope (Leica DM3000, Germany). For live cell imaging of HSA exogenously, A549 cells were incubated with HSA (1 and 4 μM) in serum free DMEM media for 24 h. Then, cells were washed with PBS buffer (pH 7.4) for three times. Subsequently, Cells were treated with 2 μM DCI-MIN for 30 min and LysoTracker dye for 10 min in serum free DMEM media. Next, the cells were washed with PBS buffer (pH 7.4) to remove unbound molecules and live fluorescence images were performed.

Urinary HSA quantification by Coomassie Brilliant Blue G250:

(A) Reagent preparation:

(1) Coomassie Brilliant Blue G250 Solution. 100 mg of Coomassie Brilliant Blue G250 was dissolved in 50 mL of 95% ethanol. To this solution 100 mL of 85% (W/V) phosphoric acid was added. Finally, the resulting solution was diluted to 1000 mL with Milli-Q water.

(2) 0.9% NaCl. 900 mg NaCl was dissolved in 100 mL Milli-Q water.

(3) HSA standard solution. A 100 mL of 0.05 mg/mL HSA standard solution was prepared by diluting the previously prepared 25 mg/mL HSA stock solution with Milli-Q water.

(B) Preparation of standard curve:

For the generation of Standard curve, 11 test tubes were taken and the reagents were added according to the following table:

Preparation of standard samples

Sample	1	2	3	4	5	6	7	8	9	10	11
HSA standard solution (mL)	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
0.9% NaCl (mL)	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0
Coomassie Brilliant Blue G250 (mL)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
HSA concentration (mg/L)	0	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0

Draw a standard addition curve with Absorbance at 595 nm versus HSA content (mg/L).

(C) Endogenous HSA determination in urine:

1 mL urine sample was taken in a fresh test tube. Then 4 mL Coomassie Brilliant Blue G250 reagent was added to react for 5 minutes. Finally, the absorbance at 595 nm was measured to determine the amount of HSA. The final data obtained by Coomassie Brilliant Blue G250 method is measured value × 5.

Estimation of HSA in Human Blood Serum.

Blood samples (5 mL each) were collected from healthy donors into a blood collecting tube using a sterilized syringe and needle. Then, the blood samples were centrifuged at 5000 rpm for 10 min to separate the serum from the red blood cells. Serum on the top portion is then pipetted out into another vial which was used for the analysis. The HSA content in blood serum was estimated with DCI-MIN by using standard addition method. Fluorescence response of DCI-MIN (2 µM) in diluted plasma sample (1000-fold dilution) upon addition of different concentrations of HSA (0−4 µM). A calibration plot was prepared by measuring the emission maximum at 602 nm (I_{602}). The unknown concentration of HSA protein in the blood serum was calculated from the calibration curve by diluting the serum sample appropriately within the linear range.

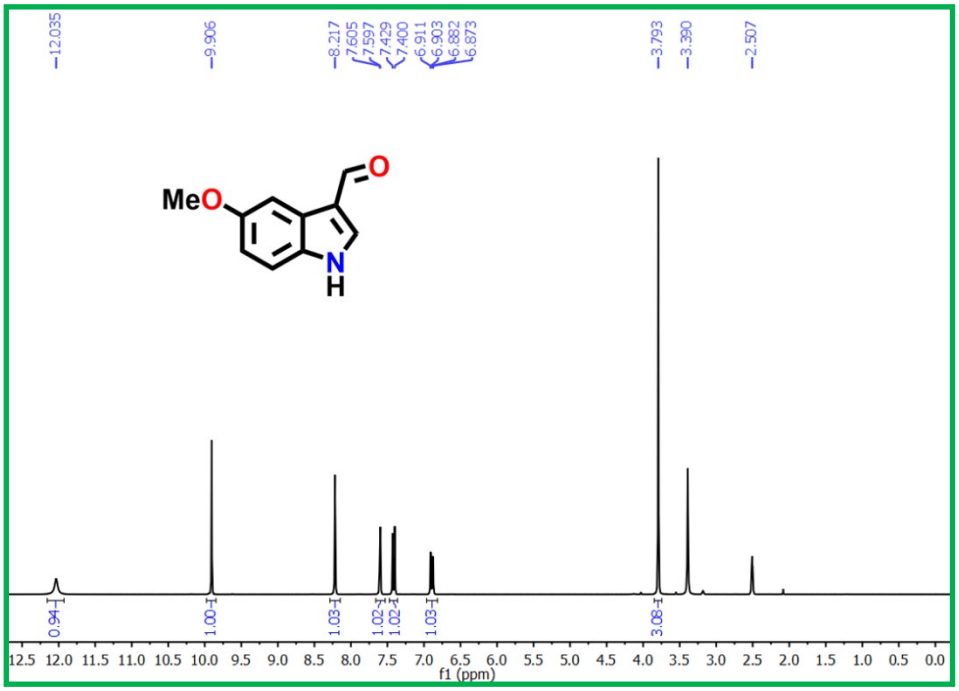


Figure S1. ^{1}H-NMR spectrum of compound MIN in DMSO-d_6.

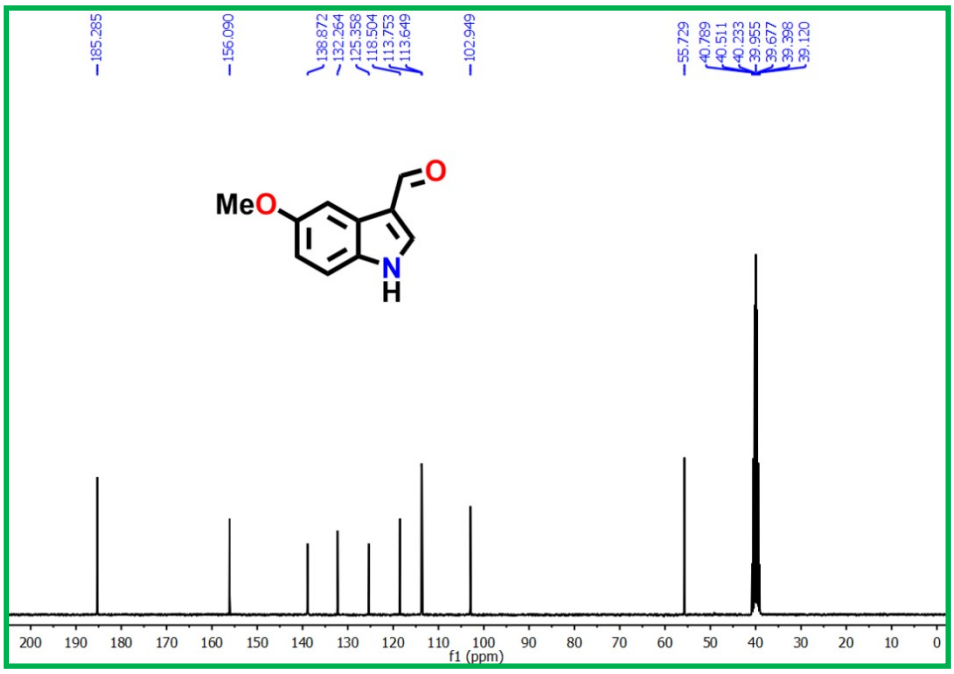


Figure S2. ^{13}C-NMR spectrum of compound MIN in DMSO-d_6.

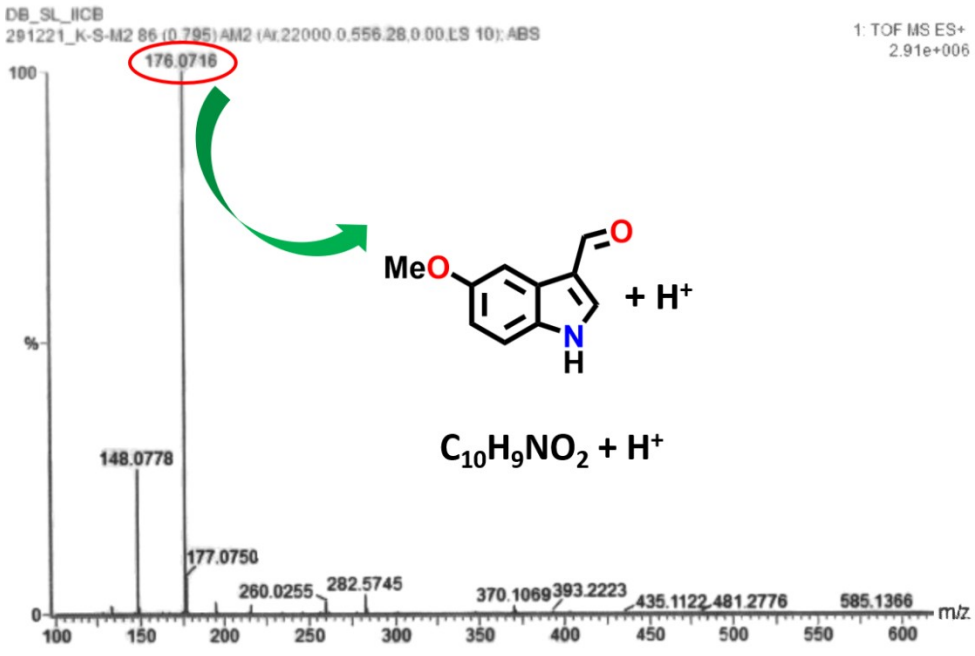


Figure S3. Mass spectrum of compound MIN in MeOH.

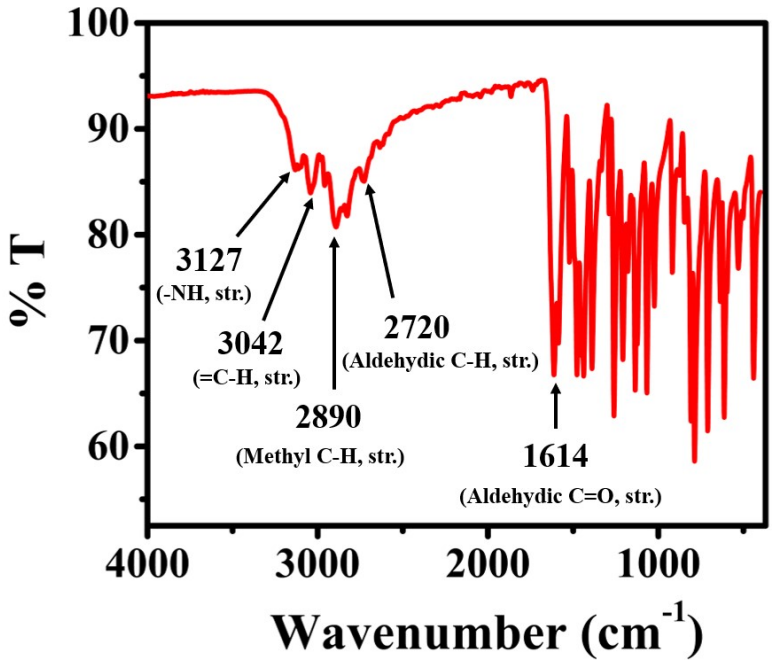


Figure S4. IR spectrum of compound MIN.

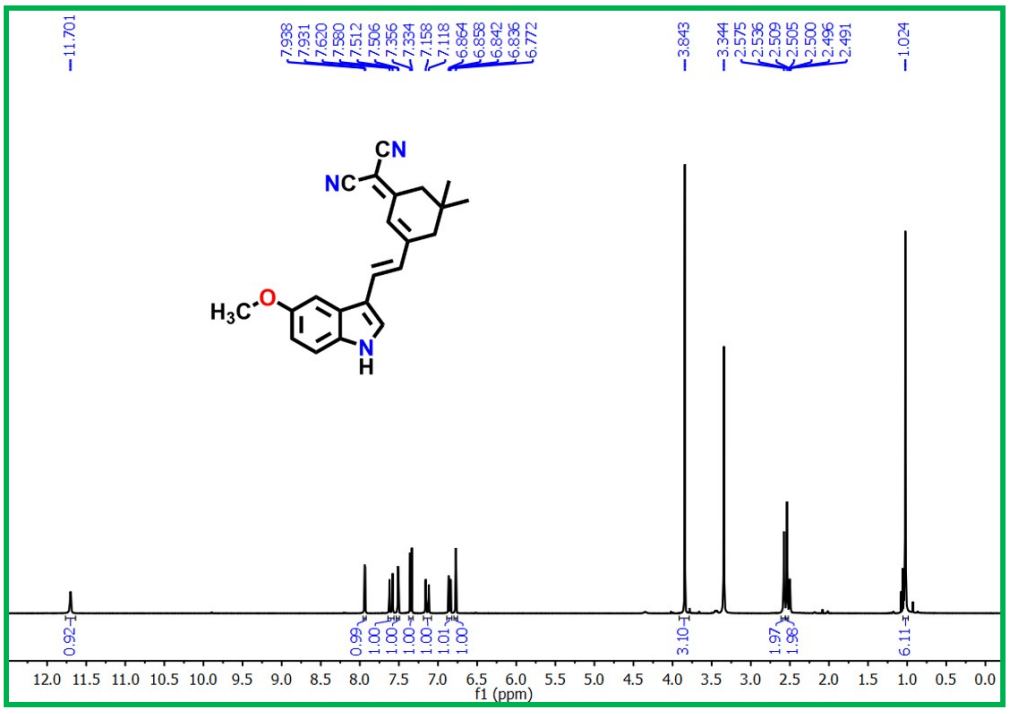


Figure S5. ^{1}H-NMR spectrum of probe DCI-MIN in DMSO-d_6.

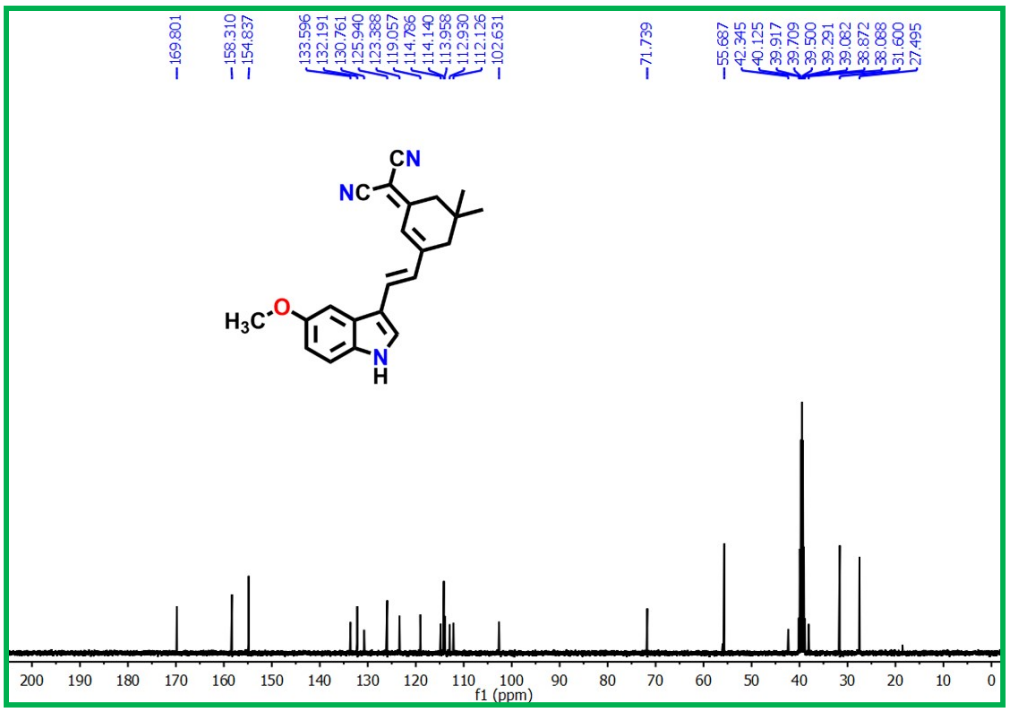


Figure S6. ^{13}C-NMR spectrum of probe DCI-MIN in DMSO-d_6.

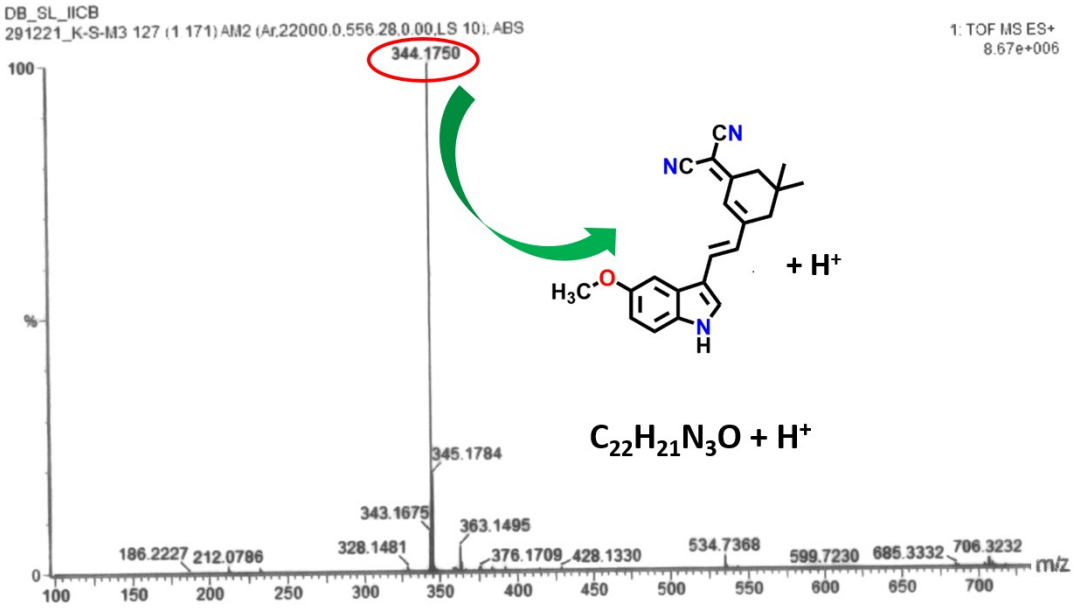


Figure S7. Mass spectrum of probe DCI-MIN in MeOH.

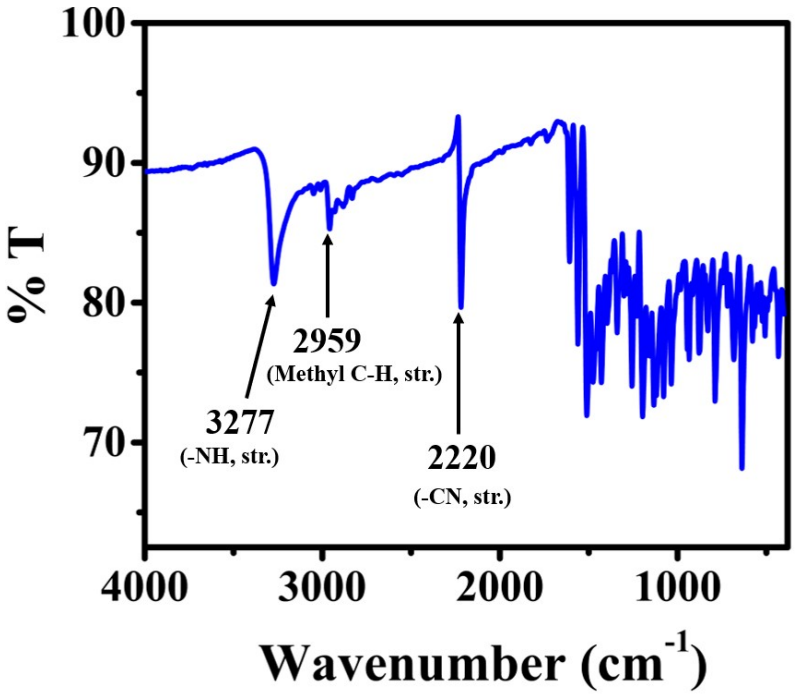


Figure S8. IR spectrum of probe DCI-MIN.

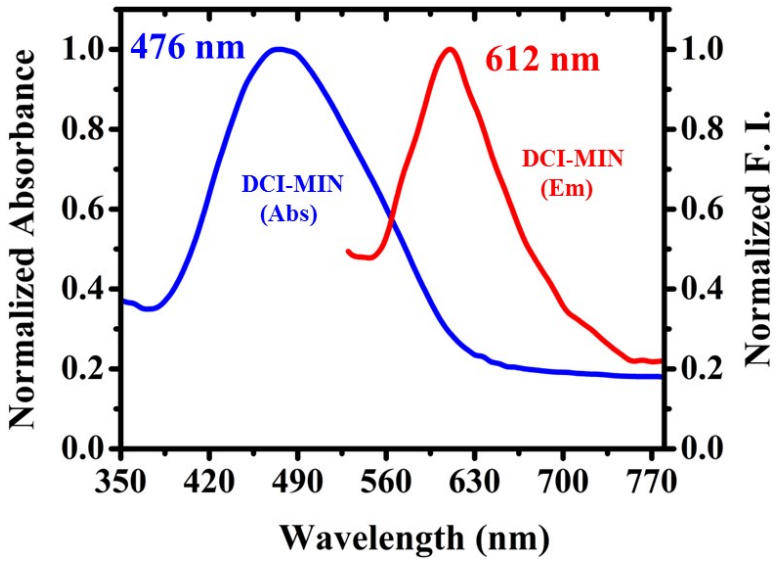


Figure S9. Normalized absorption and fluorescence spectra of DCI-MIN (2 µM) in ~100% PBS buffer (pH 7.4).

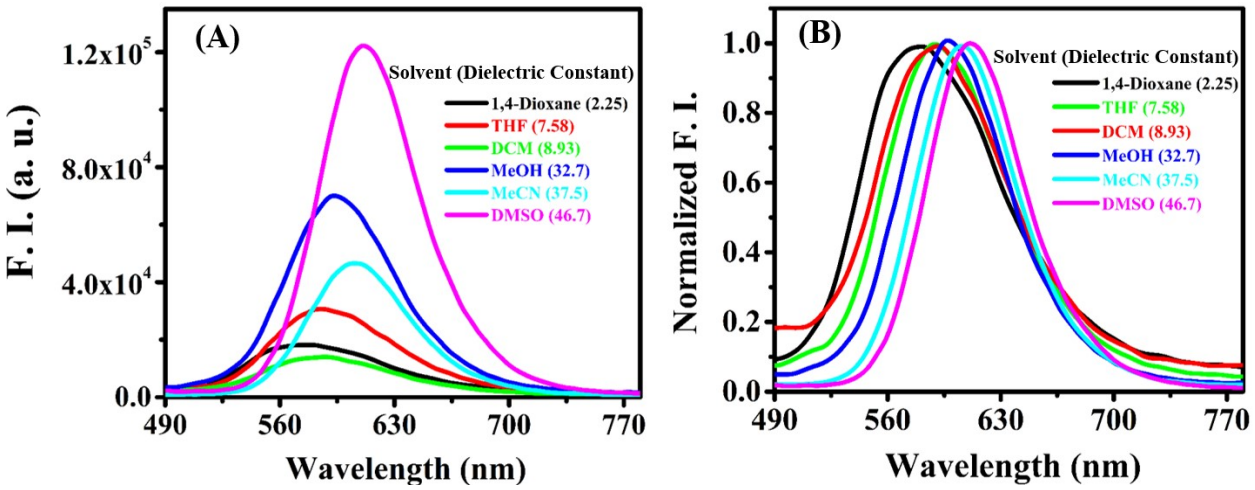


Figure S10. (A) Fluorescence spectra and **(B)** normalized fluorescence spectra of DCI-MIN (2 μM) in different solvents.

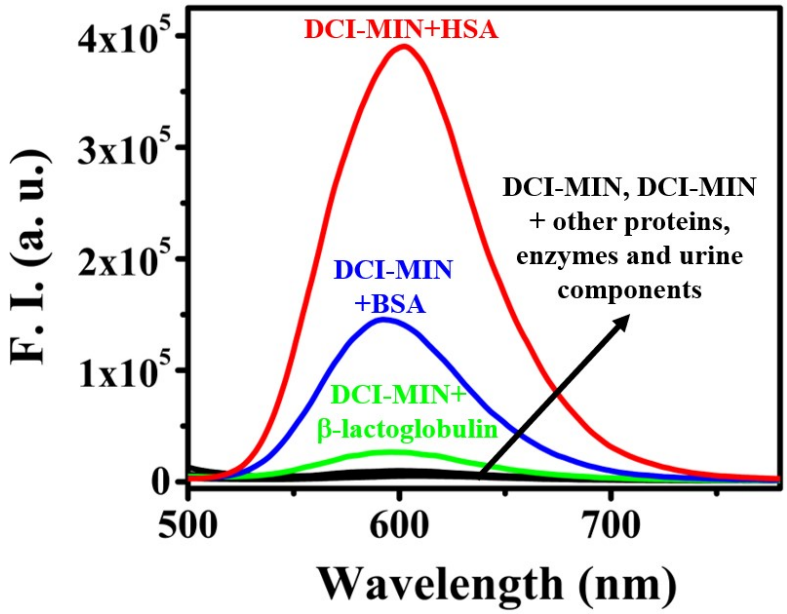


Figure S11. Fluorescence spectra of DCI-MIN (2 μM) toward various proteins, enzymes and common urine components (each at 20 μM) in PBS buffer solution of pH 7.4, λ_{ex} = 476 nm.

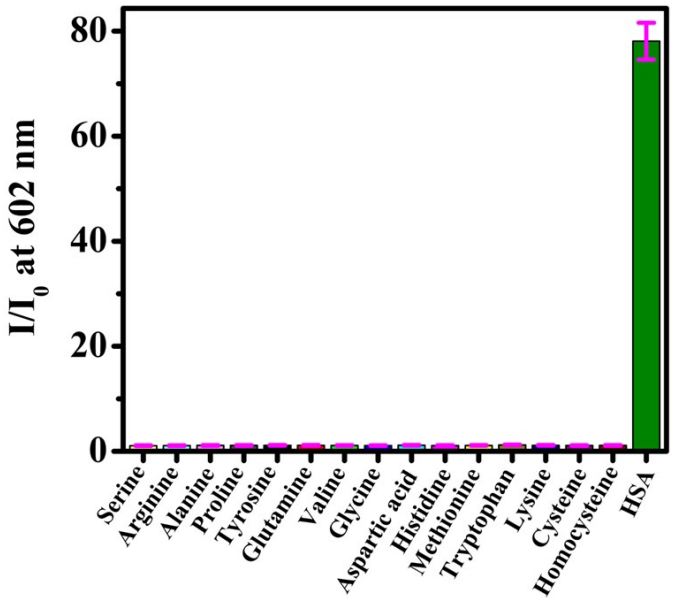


Figure S12. Emission intensity variations ($^{I/I_0}$) of DCI-MIN (2 μM) for HSA (20 μM) and various amino acids (each at 20 μM) at 602 nm in PBS buffer solution of pH 7.4, λ_{ex} = 476 nm. Error bars: standard deviation (n = 3).

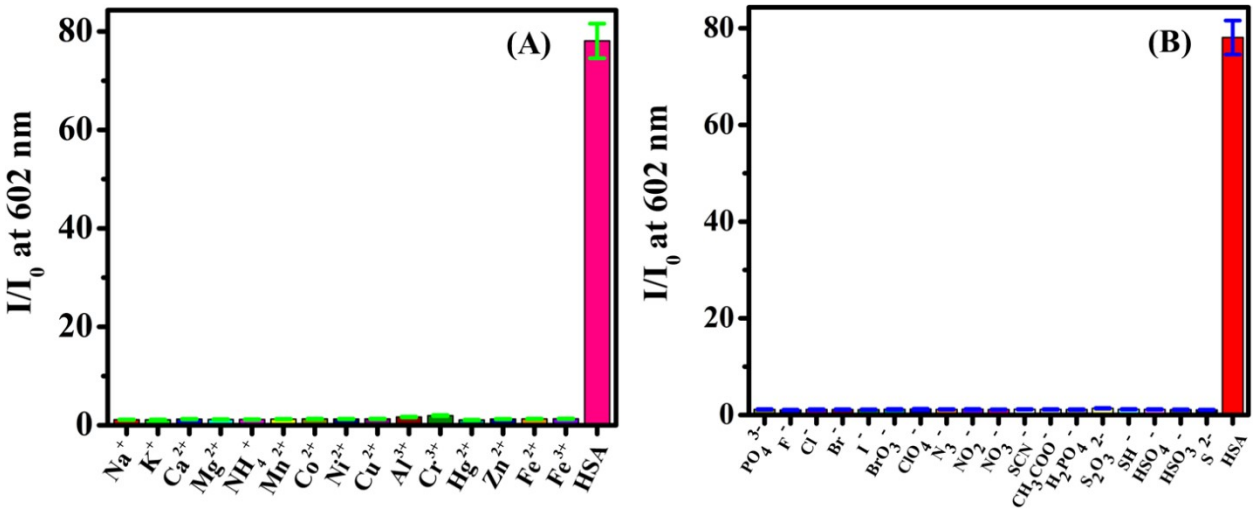


Figure S13. (A and B) Emission intensity variations ($^{I/I_0}$) of DCI-MIN (2 μM) for HSA (20 μM) and various cations and anions (each at 30 μM) at 602 nm in PBS buffer solution of pH 7.4, λ_{ex} = 476 nm. Error bars: standard deviation (n = 3).

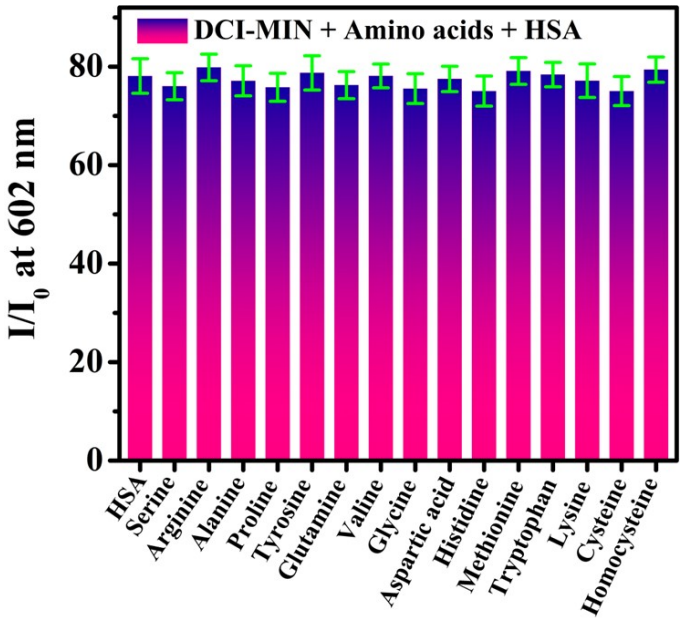


Figure S14. Emission intensity variations ($^{I}/_{I_0}$) of DCI-MIN (2 μM) for HSA (20 μM) in the presence of various amino acids (20 μM) at 602 nm in PBS buffer solution of pH 7.4, λ_{ex} = 476 nm. Error bars: standard deviation (n = 3).

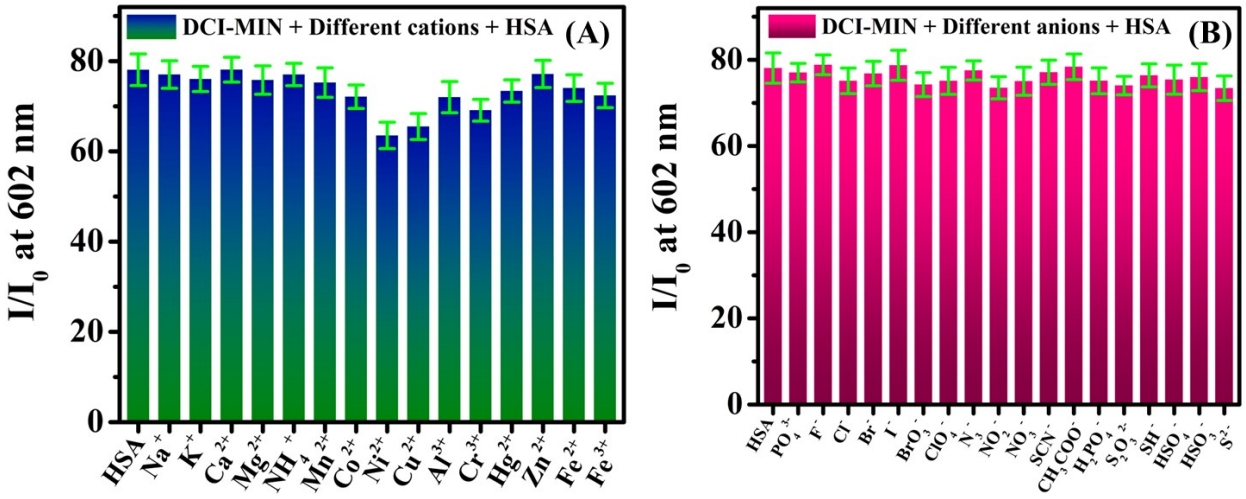


Figure S15. (A and B) Emission intensity variations ($^{I}/_{I_0}$) of DCI-MIN (2 μM) for HSA (20 μM) in the presence of different cations and anions (each at 30 μM) at 602 nm in PBS buffer solution of pH 7.4, λ_{ex} = 476 nm. Error bars: standard deviation (n = 3).

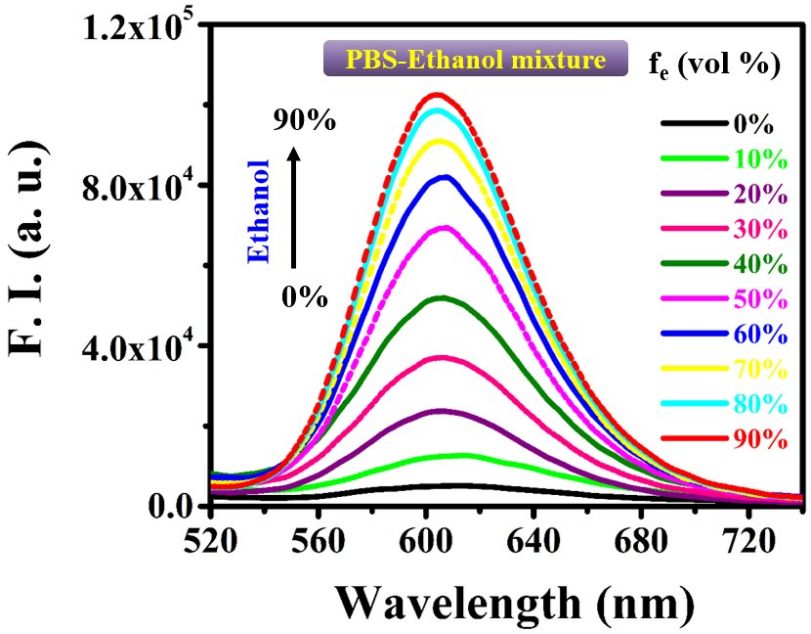


Figure S16. Fluorescence emission spectra of DCI-MIN (2 μM) in PBS/ethanol mixtures with different ethanol fractions (f_e). λ_{ex} = 476 nm.

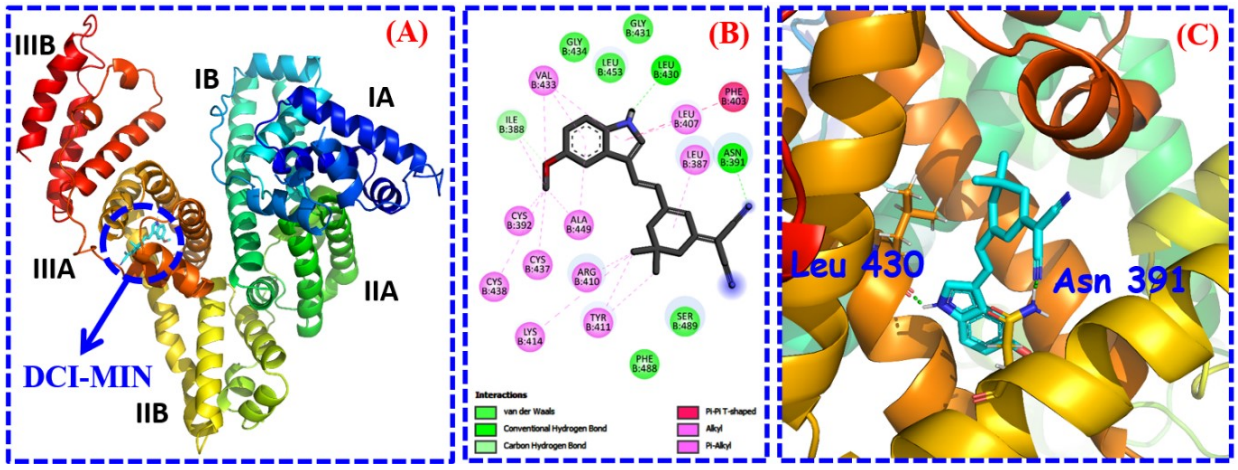
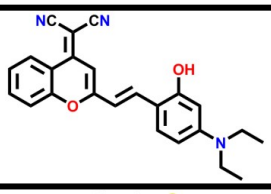
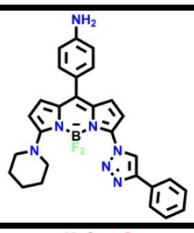
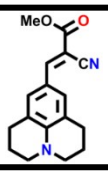
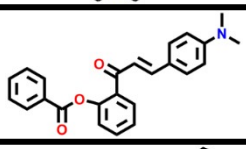
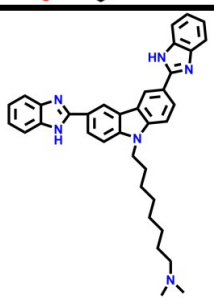
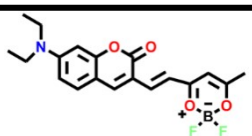
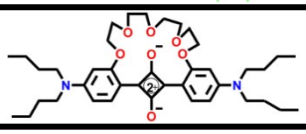
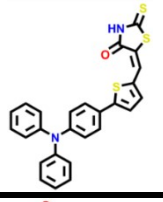
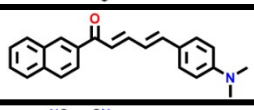
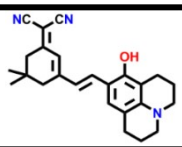
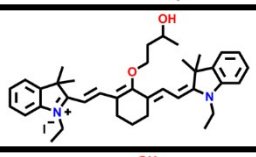
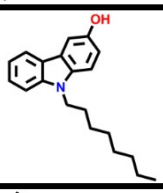
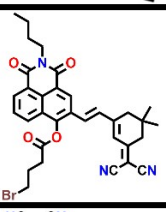
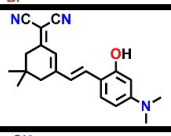
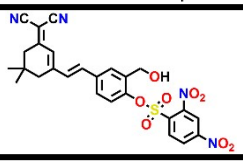
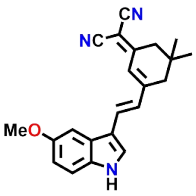


Figure S17. (A) Stereo view of docked conformation of DCI-MIN within HSA (PDB ID: 2BXE) highlighted by blue colour ring. (B) Adjacent residues and forces responsible for the binding of DCI-MIN at subdomain IIIA (site II) of HSA. (C) Enlarged view of hydrogen bonding interactions made by DCI-MIN at the binding site.

Table S1. Comparison of the fluorescent molecular probes for the detection of human serum albumin.

Structure of the probe	$\lambda_{ex}/\lambda_{em}$ (Stokes shift) (nm)	Selectivity	LOD	Response time	Binding site	Ref.
	530/650 (120)	HSA	1.26 mg/L	–	Site I	33
	436/508 (72)	HSA	5 nM	60 min	More than one site	35
	460/575 (115)	HSA	0.30 mg/L	–	Fatty acid site I	36
	440/490 (50)	HSA	0.40 mg/L	–	Site I	32
	426/524 (98)	HSA	1.91 mg/L	–	Site I	38
	350/454 (104)	HSA	0.27 mg/L	2 h	Site I	39
	495/540 (45)	HSA	0.21 mg/L	<15 min	Site I & Site II	42
	650/675 (25)	HSA	1.03 mg/L	–	Site I & Site II	44

	497/610 (113)	HSA	0.34 mg/L	~10 min	Subdomain IB	**46**
	480/610 (130)	HSA	23 nM	~3 min	Site I & Site II	**47**
	590/685 (95)	HSA	4.64 nM	~15 min	Site I	**48**
	630/710 (80)	HSA	0.996 mg/L	<5 min	Site I	**51**
	295/400 (105)	HSA	4 nM	–	Site I	**49**
	520/670 (150)	HSA	0.21 nM	13 min	Site II	**30**
	565/672 (107)	HSA	11 nM	5 min	Site I	**31**
	500/610 (107)	HSA	35 nM	–	Subdomains IIA, IIIA & IB	**56**
	476/602 (126)	HSA	1.01 nM (0.0671 mg/L)	~1.5 min	Both Subdomains IIIA (site II) & IB; but predominantly at IB	**This work**

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