SUPPORTING FILE

Design and synthesis of a TICT based red-emissive fluorescent probe for the rapid and selective detection of HSA in human biofluids and live Cell imaging

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Fig. S1 ^{1}H-NMR spectrum of QM in DMSO-d$_6$.
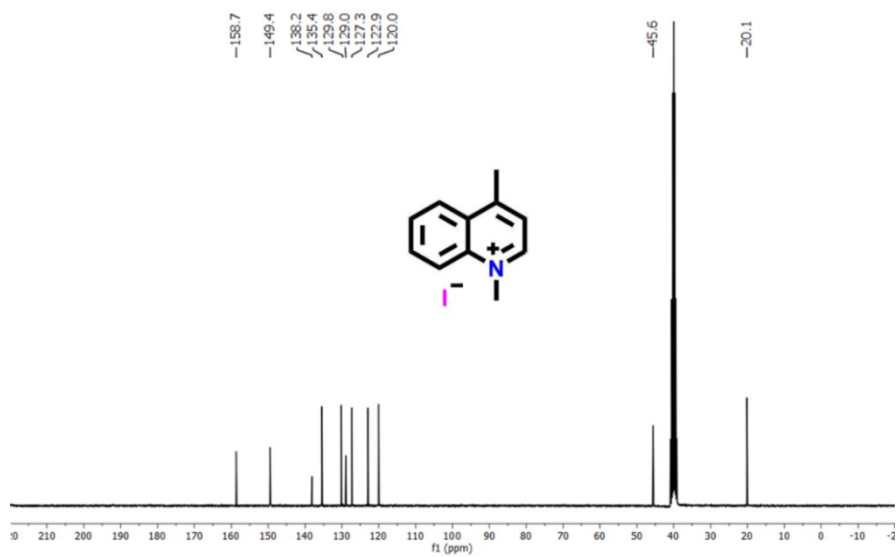
Fig. S2 ^{13}C-NMR spectrum of QM in DMSO-d$_6$.
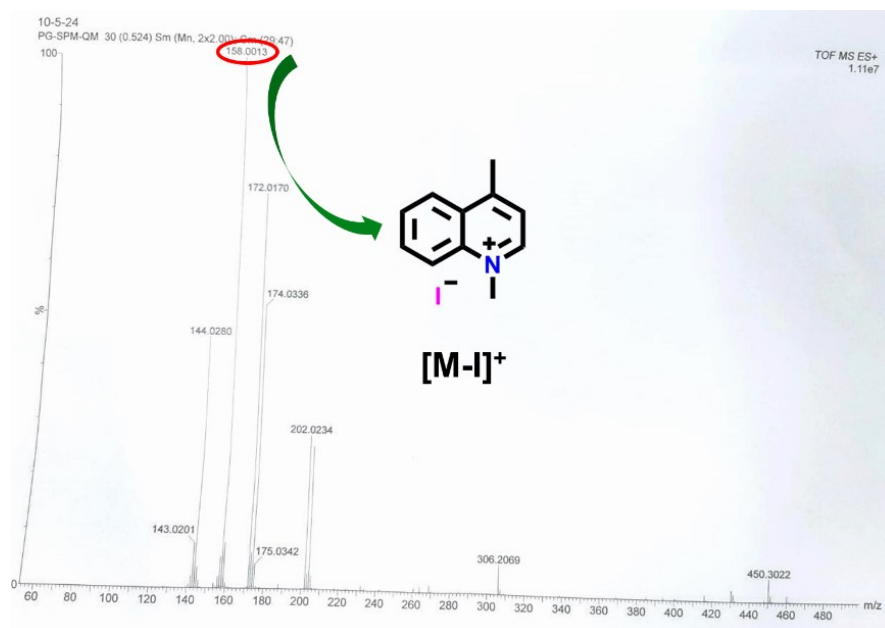
Fig. S3 Mass spectrum of probe QM in MeOH.
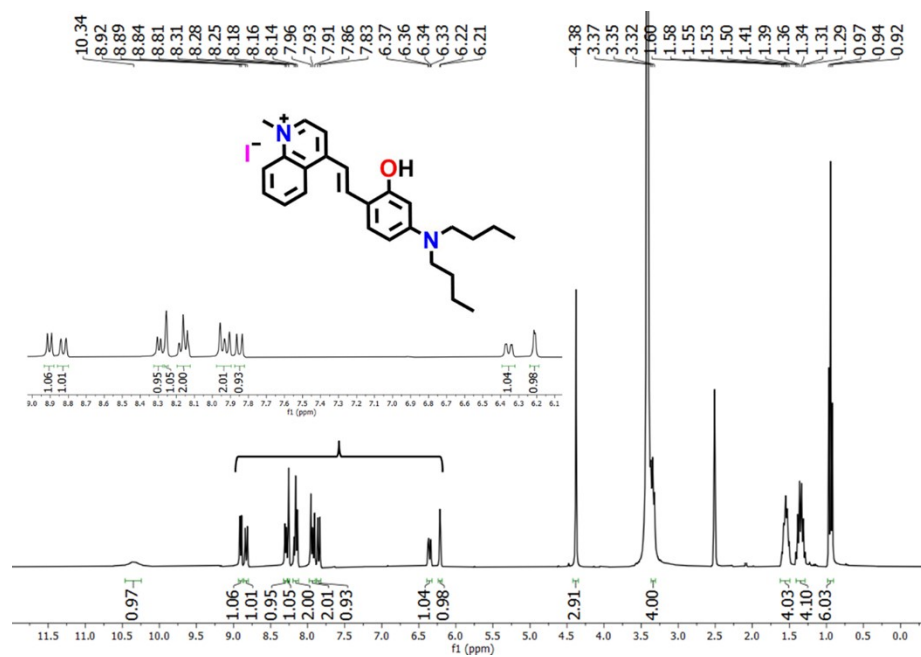
Fig. S4 ^{1}H-NMR spectrum of DHMQ in DMSO-d$_6$.
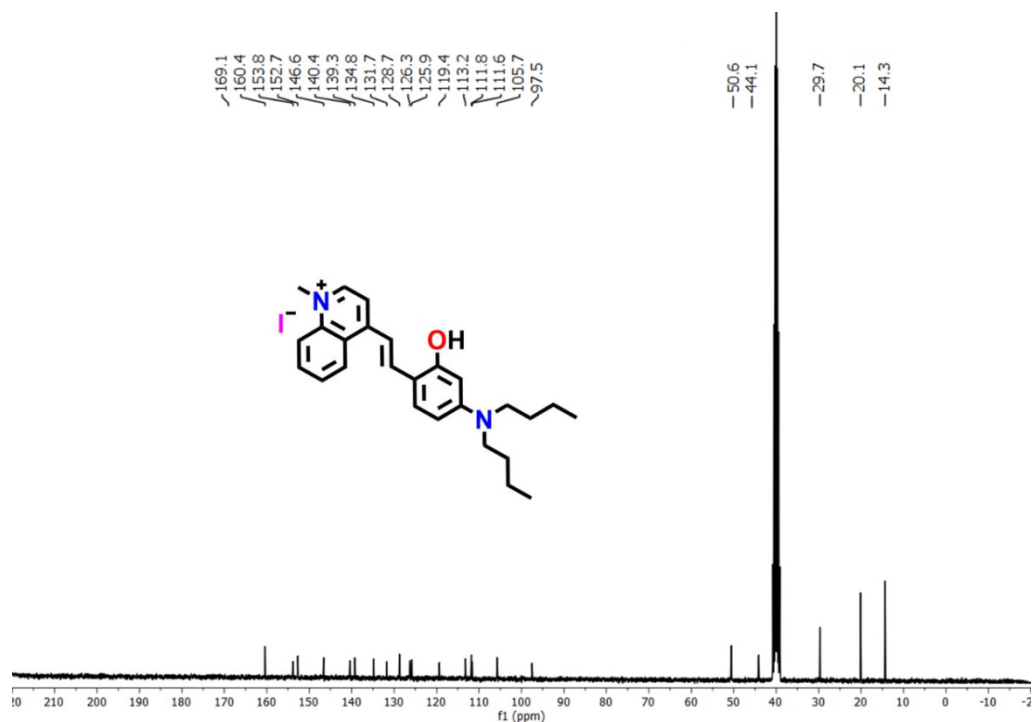
Fig. S5 ^{13}C-NMR spectrum of DHMQ in DMSO-d_6.

[M-I]$^+$
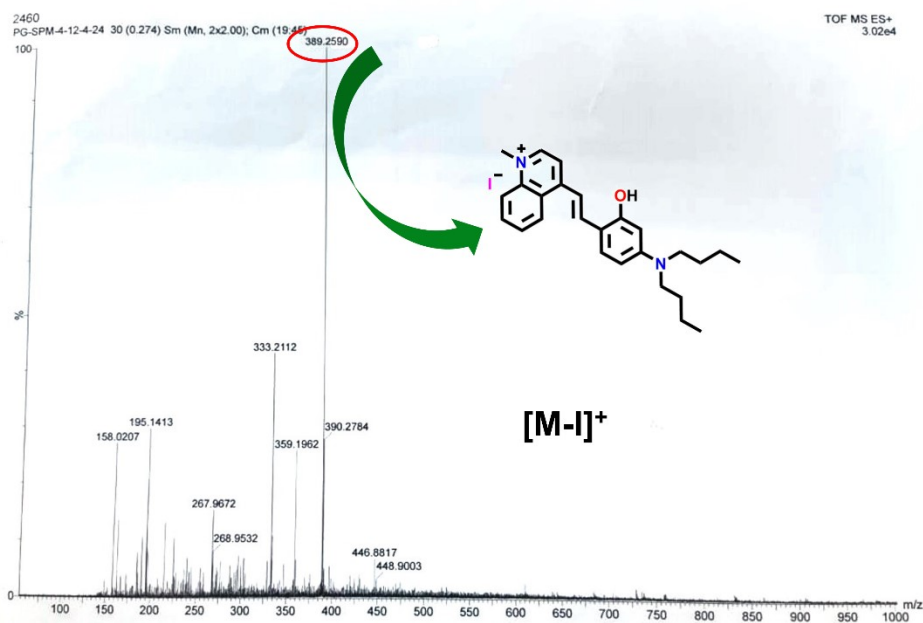
Fig. S6 Mass spectrum of probe DHMQ in MeOH.
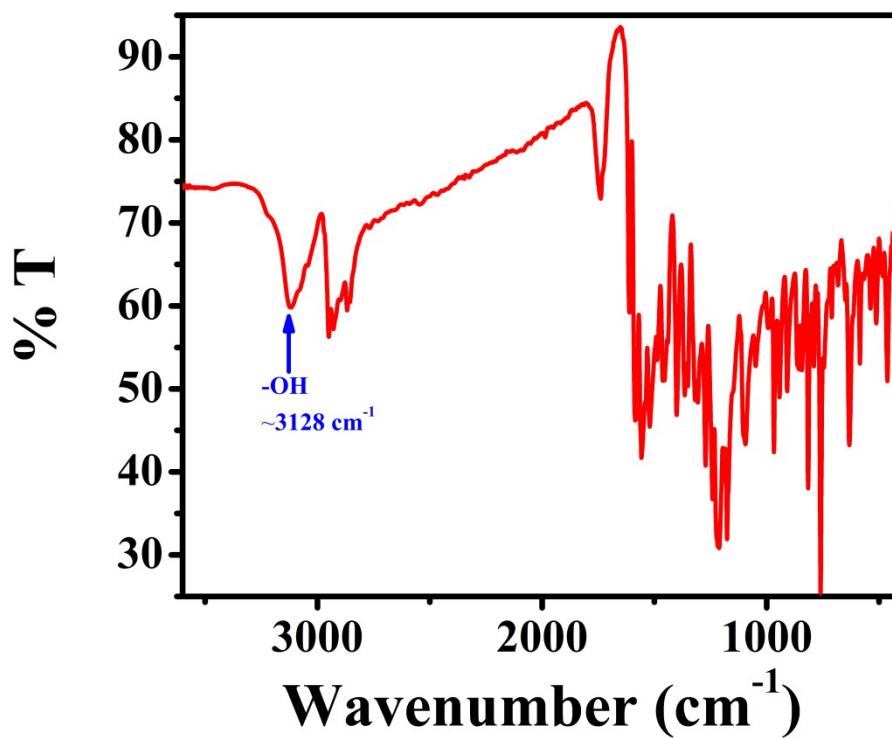
Fig. S7 IR spectrum of DHMQ.
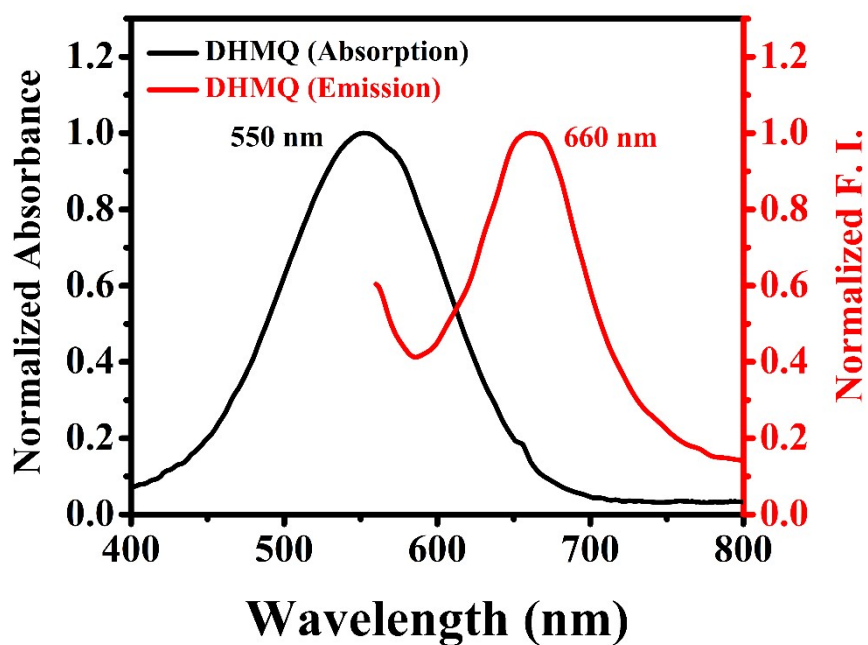
Fig. S8 Normalised absorbance and fluorescence spectra of DHMQ in ~100% PBS medium.
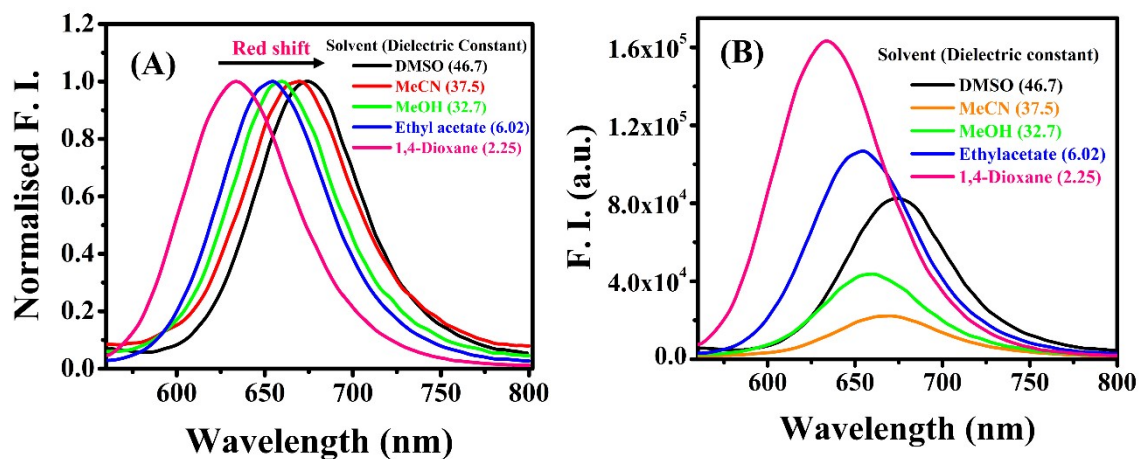
Fig. **S9** (A) Normalized fluorescence spectra of DHMQ (5 µM) in different solvents. (B) Fluorescence spectra of DHMQ (5 µM) in different solvents. λ_{ex} = 550 nm.
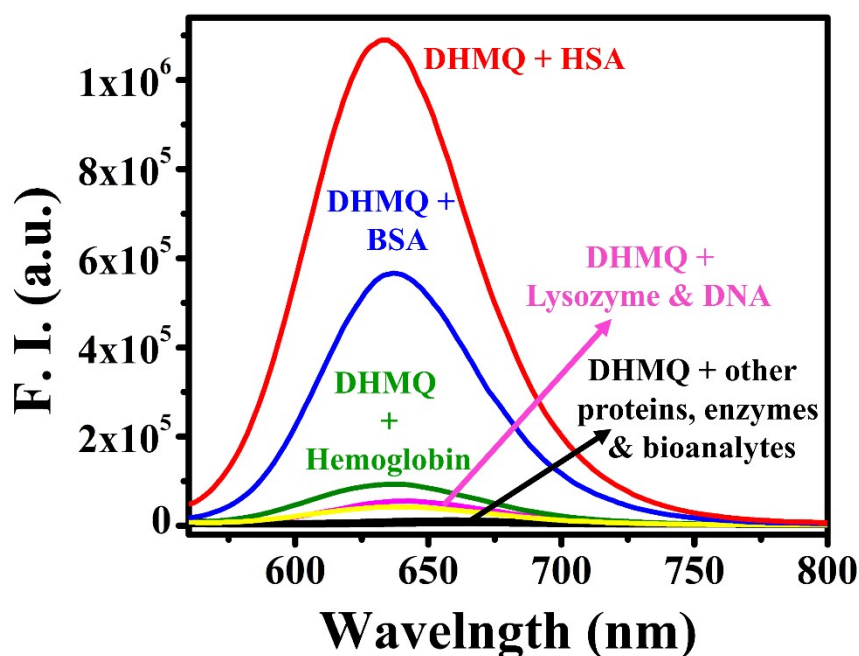
Fig. S10. Fluorescence spectra of DHMQ (5 µM) toward various proteins, enzymes and other bioanalytes (each at 8 equiv.) in ~ 100 % PBS buffer solution of pH 7.4. λ_{ex} = 550 nm.
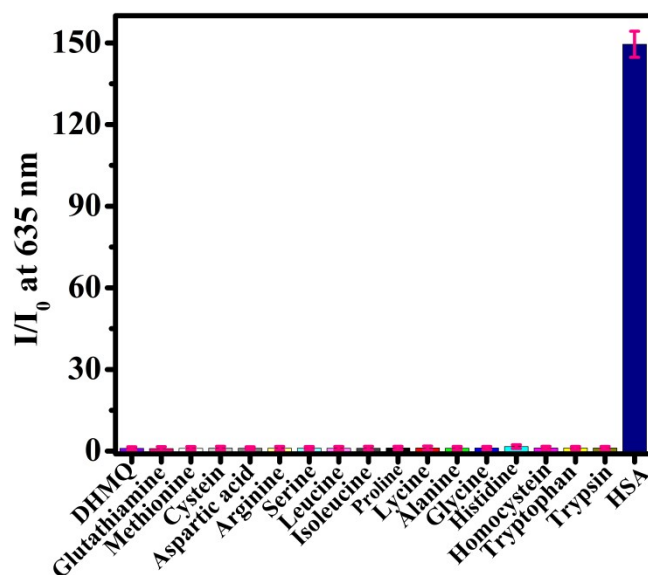
Fig. S11. Fluorescence responses of DHMQ (5 μM) at 635 nm towards HSA and various amino acids (8 equiv.) in ~100 % PBS buffer pH 7.4 at room temperature. λ_{ex}= 550 nm. Error bars: (SD, n = 3).
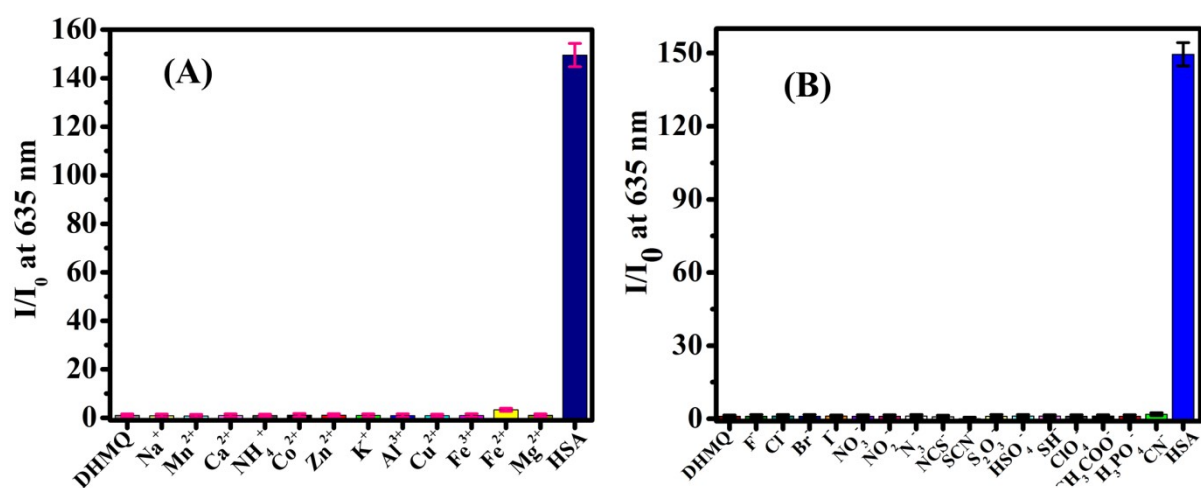
Fig. S12. (A) Fluorescence responses of DHMQ (5 μM) towards HSA and various cations (8 equiv.) at 635 nm in ~100% PBS buffer of pH 7.4. **(B)** Fluorescence responses of DHMQ (5 μM) towards HSA and various anions (8 equiv.) at 635 nm in ~ 100% PBS buffer of pH 7.4. λ_{ex}= 550 nm. Error bars: (SD, n = 3).
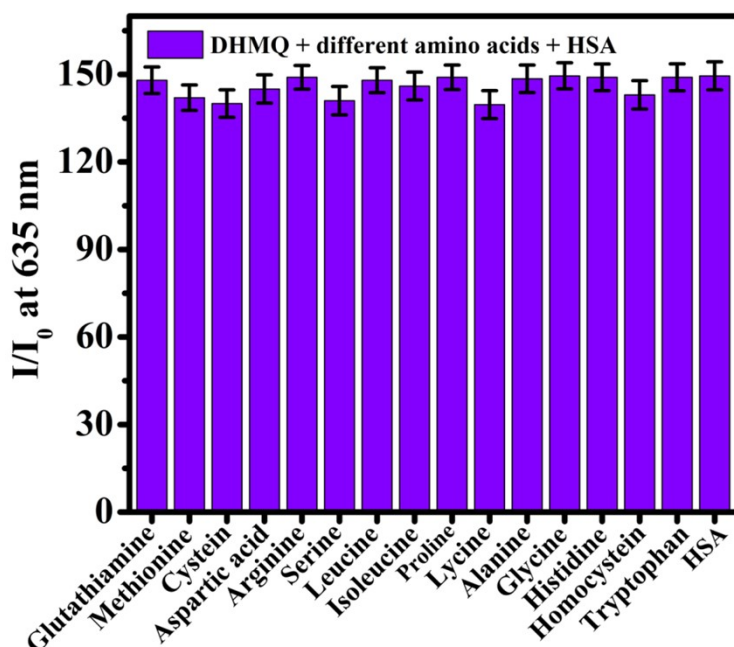
Figure S13. Fluorescence responses of DHMQ (5 µM) toward HSA in the presence of various amino acids (8 equiv.) at 635 nm in ~100% PBS buffer of pH 7.4. λ_{ex} = 550 nm. Error bars: (SD, n = 3).
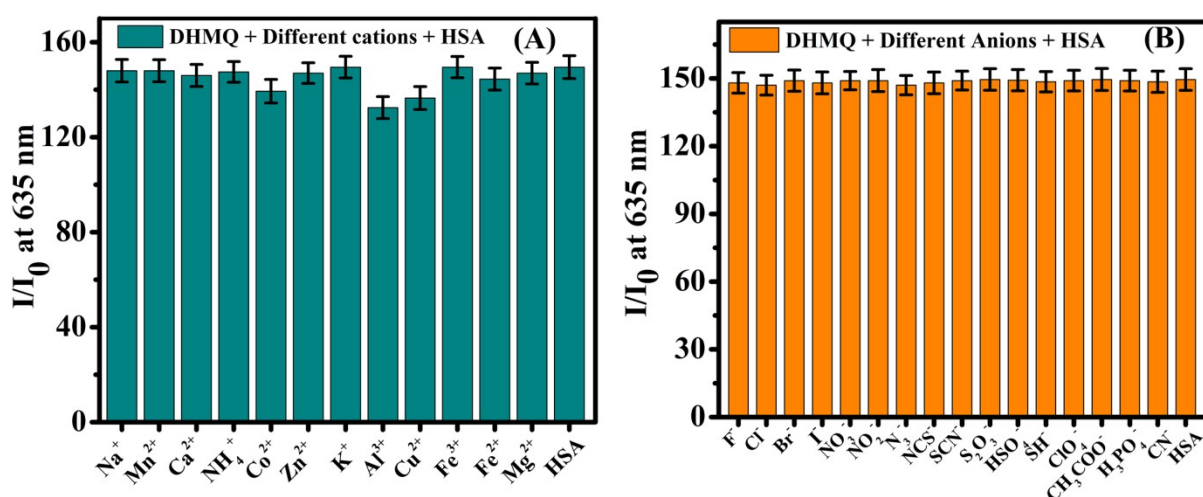
Figure S14. Fluorescence responses of DHMQ (5 µM) toward HSA in the presence of various cations (8 equiv.) at 635 nm in ~100% PBS buffer of pH 7.4. (**B**) Fluorescence responses of DHMQ (5 µM) toward HSA in the presence of various anions (8 equiv.) at 635 nm in ~100% PBS buffer of pH 7.4. λ_{ex} = 550 nm. Error bars: (SD, n = 3).
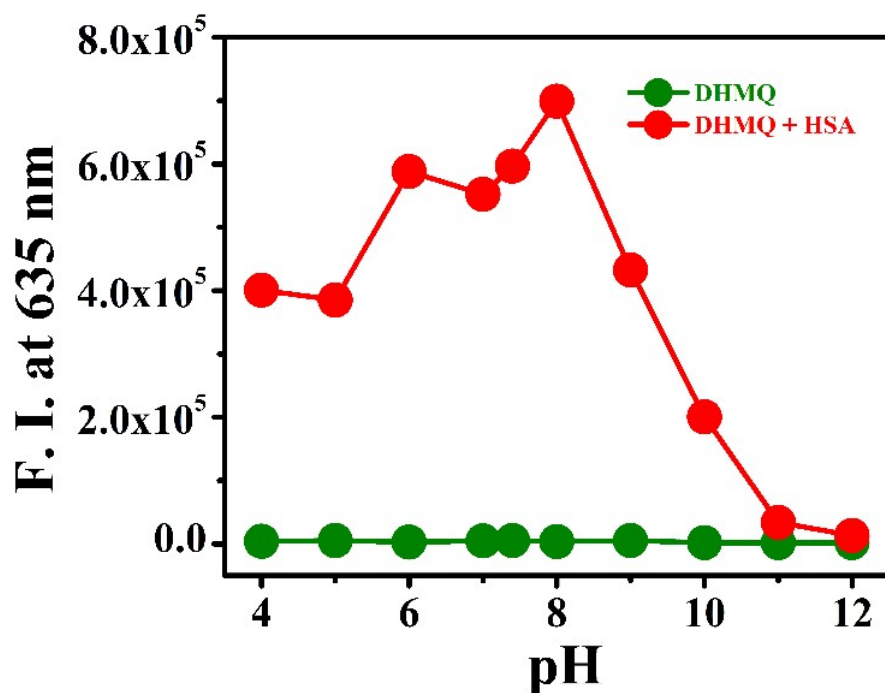
Figure S15. The fluorescence emission intensities of **DHMQ** (5 µM) at 635 nm in the absence and presence of HSA (1 equiv.) at different pHs. All the experiments were performed in PBS buffer. λ_{ex} = 550nm.
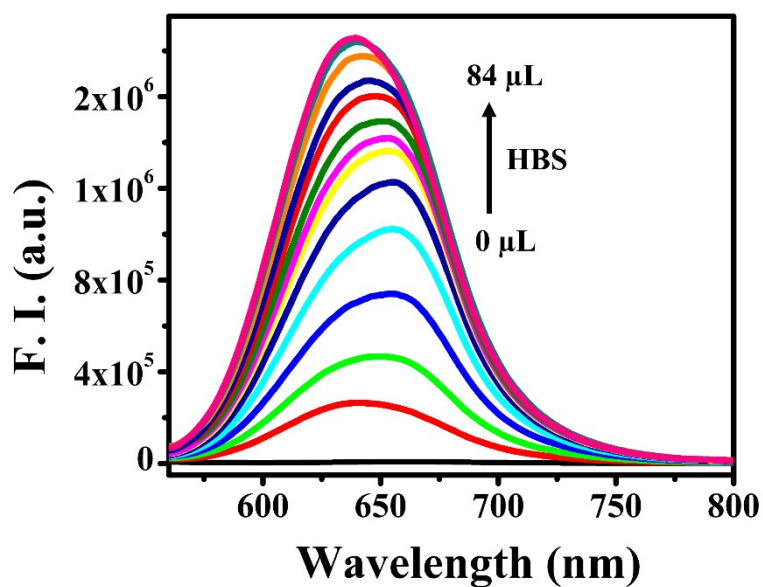
Fig. S16 Change of emission intensity of DHMQ (5 µM) in ~100% PBS buffer, pH 7.4 at 639 nm (λ_{ex} =550 nm) upon addition of aliquots (0 - 84 µL) of human blood serum (HBS).

Instrumentation

The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 300 MHz spectrophotometer using tetramethylsilane as internal standard in DMSO-d_6. The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, dd = doublet of doublet, m = multiplet. The ESI-MS$^+$ (m/z) spectra of the probe was recorded on a HRMS spectrophotometer (model: QTOF Micro YA263). The Fourier transform infrared spectra (4000–400 cm^{-1}) of the probe was recorded 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 Spectro fluorimeter (Model QM-40) by using a fluorescence free quartz cuvette of 1 cm path length. The excitation and emission slit widths were fixed at 5 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

For various spectroscopic studies a stock solution of DHMQ (1×10^{-3} M) was prepared in DMSO from which 10 μL was added to 2 mL of PBS buffer solution of pH 7.4 to get final concentration of 5 μM. In the fluorescence selectivity experiment, the test samples were prepared by adding the appropriate volume of the stock solutions of the respective proteins, enzymes, cations, anions and other bio-analytes into 2 mL solution of the probe DHMQ (5 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. For the fluorescence-titration, Quartz cuvette was filled with 2 mL of PBS buffer containing 5 μM DHMQ, to which the newly prepared stock solutions of HSA (1 × 10^{-4} M) were gradually added using a micropipette as required. For the fluorescence experiments, excitation wavelength was set at 550 nm and emissions were recorded from 560 to 800 nm. For the UV-Vis studies the probe concentration was also kept fixed at 5 μM and the spectra were collected with proper background correction. In case of site marker displacement assay, HSA solution was premixed with DHMQ at a molar ratio 1:4. Then, this mixed solution was further spiked with different amounts of warfarin, ibuprofen or hemin and the resultant ternary mixtures were subjected for the fluorescence measurement.

Steady-state Fluorescence Anisotropy

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

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

(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

$$G = \frac{I_{HV}}{I_{HH}}$$

(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 550 nm and 635 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 DHMQ in the absence and presence of increasing concentration of HSA at 25 °C. During the TCSPC measurements the photoexcitation was fixed at 55- nm. Here, sodium dodecyl sulphate is used as the IRF of the exciting source in the TCSPC measurement. The fluorescence decay curves were fitted to a biexponential function:

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

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

Fluorescence Quantum Yield Measurements

Fluorescence quantum yield was calculated by adopting the reported strategy [S3] where relative measurement was carried out using Rhodamine B as reference (Φ_S =0.89 in ethanol) [S4] and by considering the following equation:

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

Where, $"\Phi"$ 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 standard deviation, σ, of the blank (**DHMQ** only) was determined by measuring the fluorescence intensity 10 times separately at a fixed concentration of 5 μM. Then, the fluorescence emission at 635 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: [S5]

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 know the probable binding sites within HSA and the binding mode of DHMQ with HSA, molecular docking study was performed using docking program Auto Dock (version 4.2). The X-ray crystal structure of HSA was taken from RCSB Protein Data Bank having PDB ID: 6m4r. To draw the structure of DHMQ, Chem3D Ultra 12.0 was used and further modification was carried out by using Gaussian 09W and Auto Dock 4.2 programs. Gasteiger charges and polar hydrogen atoms were added to the protein and probe. Grid box with dimensions of 40 Å × 40 Å × 40 Å and 0.375 Å grid spacing with coordinate of centre Grid box (-31.867, -7.414, 9.181) were

specified to enclose the protein using Auto Grid program. The default values shown by the Auto Dock program were used for other sets of parameters. The Lamarckian genetic algorithm (LGA) was used to accomplish docking calculations and the grid maps for energy were calculated by AutoGrid.[S6, S7] The best optimized docked model with lowest binding energy was considered for further study of docking simulations and the output was best viewed by using Discovery Studio.

Estimation of HSA in Human Blood Serum.

Blood samples (5 mL each) were collected from healthy donors into a blood collecting tube using sterilized syringe and needle. The blood samples were allowed to clot by leaving it undisturbed at room temperature for 10–20 min. The blood samples were centrifuged at 4000 rpm for 15 min at 4 °C 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 DHMQ probe by using standard addition method. A calibration plot was prepared by measuring the emission maximum at 635 nm (I_{635}) upon addition of different concentration of HSA (0 to 1.18×10^{-6} M) to the DHMQ (5×10^{-6} M). 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.

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.
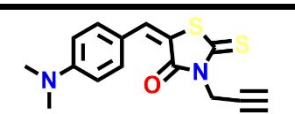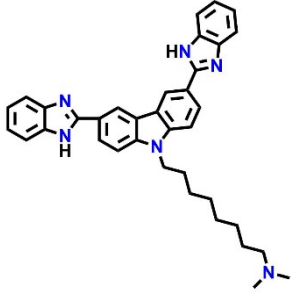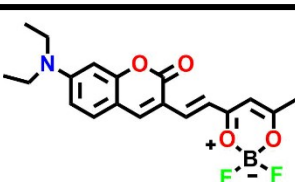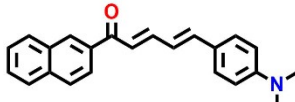
Cell Cytotoxicity assay

Cell viability of DHMQ was assessed using the MTT assay. A549 cells were cultured in 24-well plates with DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C with 5% CO2. After treatment with various DHMQ doses for 24 hours, fresh media were added. Then, 50 µL of a 5 mg/mL MTT aqueous solution was added to each well and incubated for 4 hours. The resultant purple formazan was dissolved in a 1:1 mixture of DMF-water and DMSO, and the absorbance was measured at 570 nm using a microplate reader. Relative cell viability was calculated assuming 100% viability for cells without DHMQ.
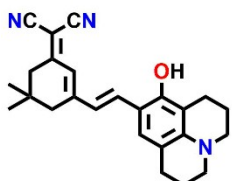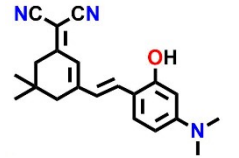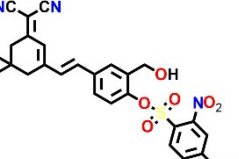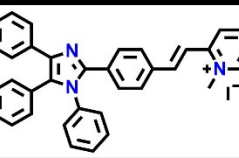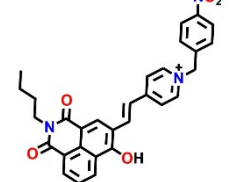
Cell Imaging Study

A549 cells were cultured on glass cover slips in 24-well plates with DMEM containing 10% FBS and 1% penicillin-streptomycin. After overnight incubation, the cells were serum-starved by replacing the medium with DMEM lacking 10% FBS for 6 hours. For cell imaging after exposure to DHMQ, cells were treated with 5 µM DHMQ in serum-free DMEM for 30 minutes, followed by three washes with PBS buffer (pH 7.4). Imaging was performed using a fluorescence microscope (Leica DM3000, Germany). For HSA imaging, A549 cells were incubated overnight with varying concentrations of HSA (5, 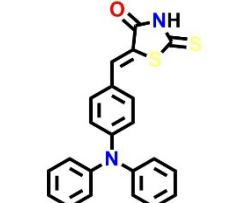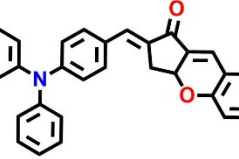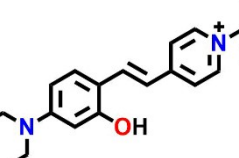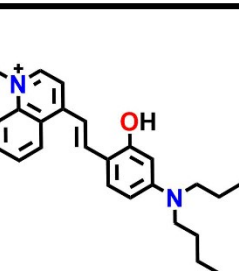10, and 20 µM) in serum-free DMEM. After washing three times with PBS buffer, the cells were treated with 5 µM DHMQ for 30 minutes in serum-free DMEM, followed by fluorescence imaging.

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.
	436/508 (72)	HSA	5 nM	60 min	More than one site	**40**
	350/454 (104)	HSA	0.27 mg/L	2 h	Site I	**41**
	495/540 (45)	HSA	0.21 mg/L	<15 min	Site I & Site II	**46**
	480/610 (130)	HSA	23 nM	~3 min	Site I & Site II	**49**

Structure	λex/λem (nm) (Φ)	Protein	K_d	Response time	Binding site	Ref.
	590/685 (95)	HSA	4.64 nM	~15 min	Site I	**66**
	565/672 (107)	HSA	11 nM	5 min	Site I	**42**
	500/610 (107)	HSA	35 nM	-	Subdomains IIA, IIIA & IB	**74**
	389/540 (151)	HSA	0.06 µM	-	Subdomain IB	**S8**
	500/630 (130)	HSA	0.246 µg/mL	~5 sec	Subdomain IB	**S9**
	485/575 (90)	HSA	0.98 µg/mL	4 min	Subdomain IB	**S10**
	485/579 (94)	HSA	13.65 µg/mL	~ 10 min	Subdomain IB	**36**
	480/580 (100)	HSA	4.8 nM	~30 sec	Site I	**31**
	550/635 (85)	HSA	0.158 mg/L	Instant	Subdomain IB	**Present work**

SUPPORTING FILE

References

1. A. Larsson, C. Carlsson, M. Jonsson, B. Albinsson, *J. Am. Chem. Soc.* 1994, **116**, 8459–8465.
2. M. Sasmal, R. Bhowmick, A. S. M. Islam, S. Bhuiya, S. Das, M. Ali, *ACS Omega,* 2018, **3**, 6293–6304.
3. A. M. Brouwer, *Pure Appl. Chem.* 2011, **83**, 2213–2228.
4. J. Hou, M. Qian, H. Zhao, Y. Li, Y. Liao, G. Han, Z. Xu, F. Wang, Y. Song, Y. Liu, *Analytica Chimica Acta*, 2018, **1024**, 169-176.
5. T. Zhu, J. Du, W. Cao, J. Fan, X. Peng, *Ind. Eng. Chem. Res.* 2016, **55**, 527-533.
6. G. M. Morris, D. S. Goodsell, R. Huey, A. J. Olson, Distributed Automated Docking of Flexible Ligands to Proteins: Parallel Applications of AutoDock 2.4. *J. Comput.-Aided Mol. Des.* 1996, **10**, 293–304.
7. G. M. Morris, D. S. Goodsell, R. Huey, A. J. Olson, Distributed Automated Docking of Flexible Ligands to Proteins: Parallel Applications of AutoDock 2.4. *J. Comput.-Aided Mol. Des.* 1996, **10**, 293–304.
8. Y. Luo, Q. Q. Yu, J. J. Gao, X. X. Lang, H. Y. Li, X. F. Yu, X. Y. Qi and M. Q. Wang, *Bioorg. Med. Chem. Lett.*, 2021, **53**, 128438.
9. M. Zhang, J. Cao, C. Huang, M. Liu, Y. Li, C. Wang, Y. Tu, *Dyes and Pigm.*, 2023, ***208***, 110867.
10. S. Pei, J. Li, N. Kang, G. Zhang, B. Zhang, C. Zhang, and S. Shuang, *Anal. Chim. Acta.*, 2022, **1190**, 339267.