"Turn-On" monopodal and dipodal nanoprobes for serum albumins – A case of shift in selectivity towards BSA and Z- to U-like conformational change

Sukhvinder Dhiman[a], Rasdeep Kour[b] Gulshan Kumar[c], Satwinderjeet Kaur[b], Vijay Luaxmi[d], Prabhpreet singh[a], Subodh Kumar[a*]

[a*]Department of Chemistry, Center for Advanced Studies, Guru Nanak Dev University, Amritsar, Punjab, India
[b]Department of Botany and Environment Science, , Guru Nanak Dev University, Amritsar, Punjab, India
cDepartment of Chemistry, M.M. Engineering College, Maharishi Markandeshwar University, Mullana-133207, India
[d]School of Chemistry and Biochemistry, Thapar Institute of Engineering and Technology, Patiala, Punjab, India

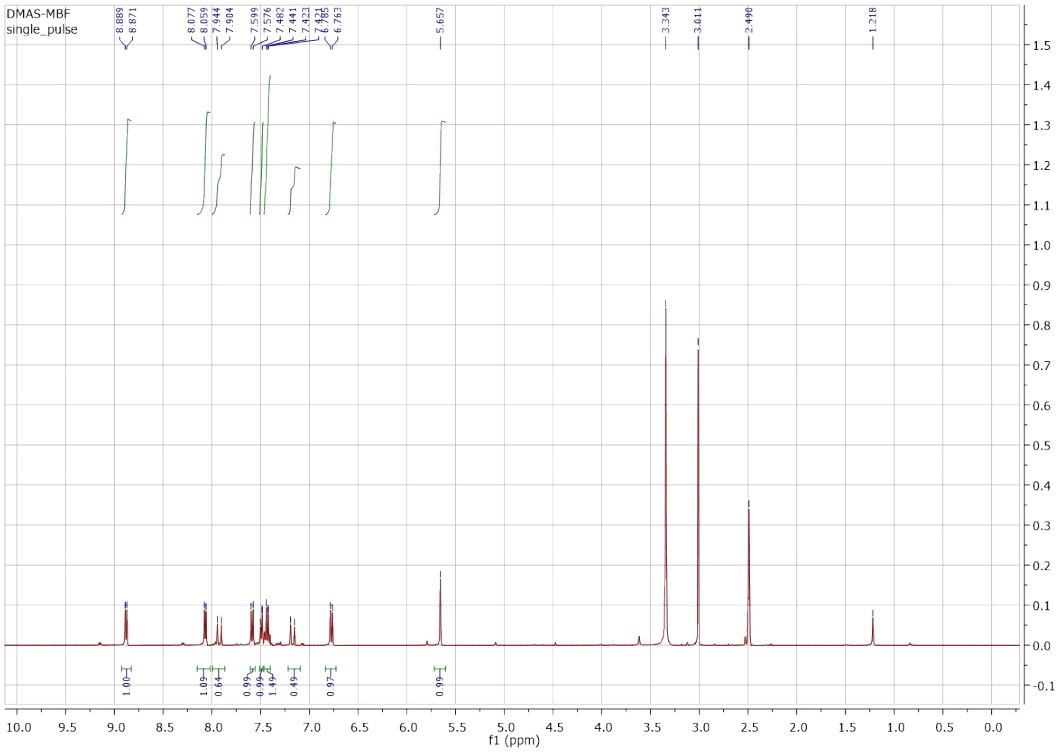

Figure: S1 ^{1}H NMR spectrum of MPMB

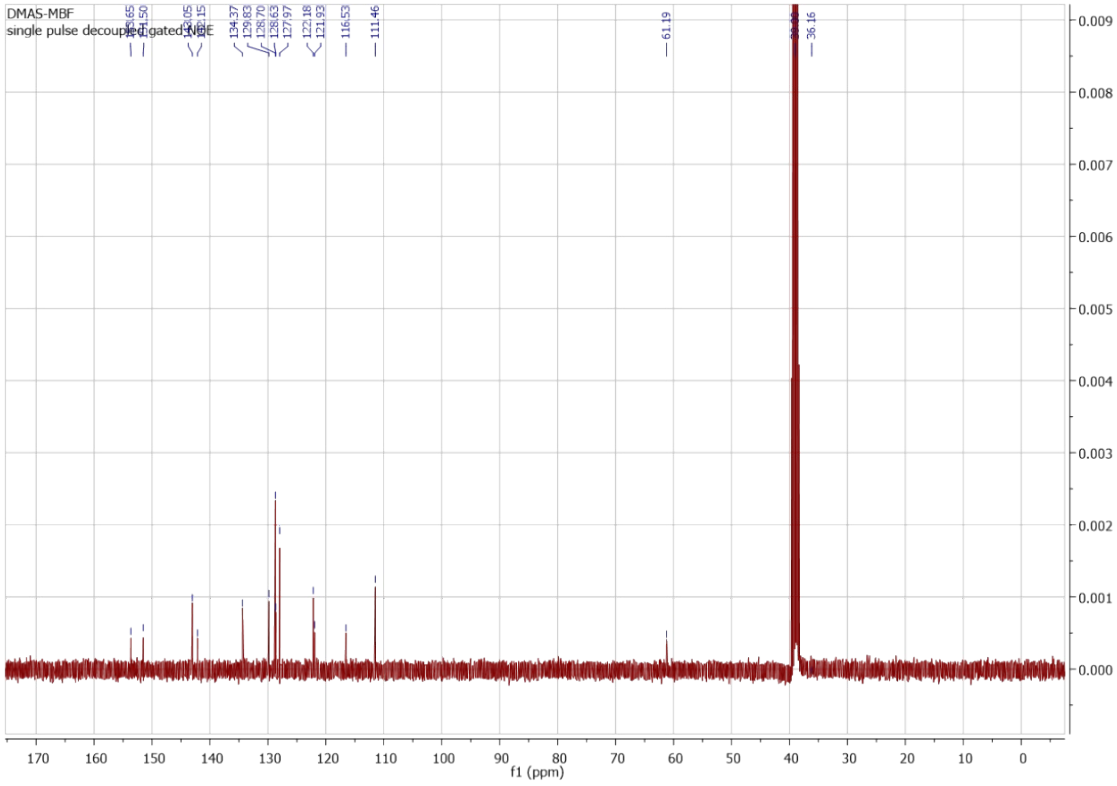

Figure: S2 ^{13}C NMR spectrum of MPMB

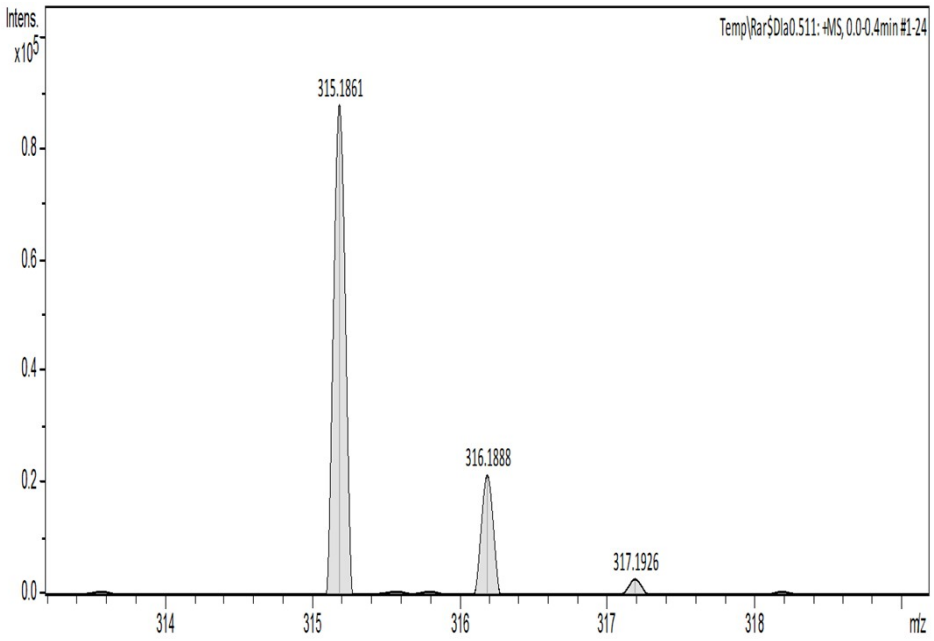

Figure S3: HRMS spectrum of MPMB indicating fragment at $C_{22}H_{33}N_2$

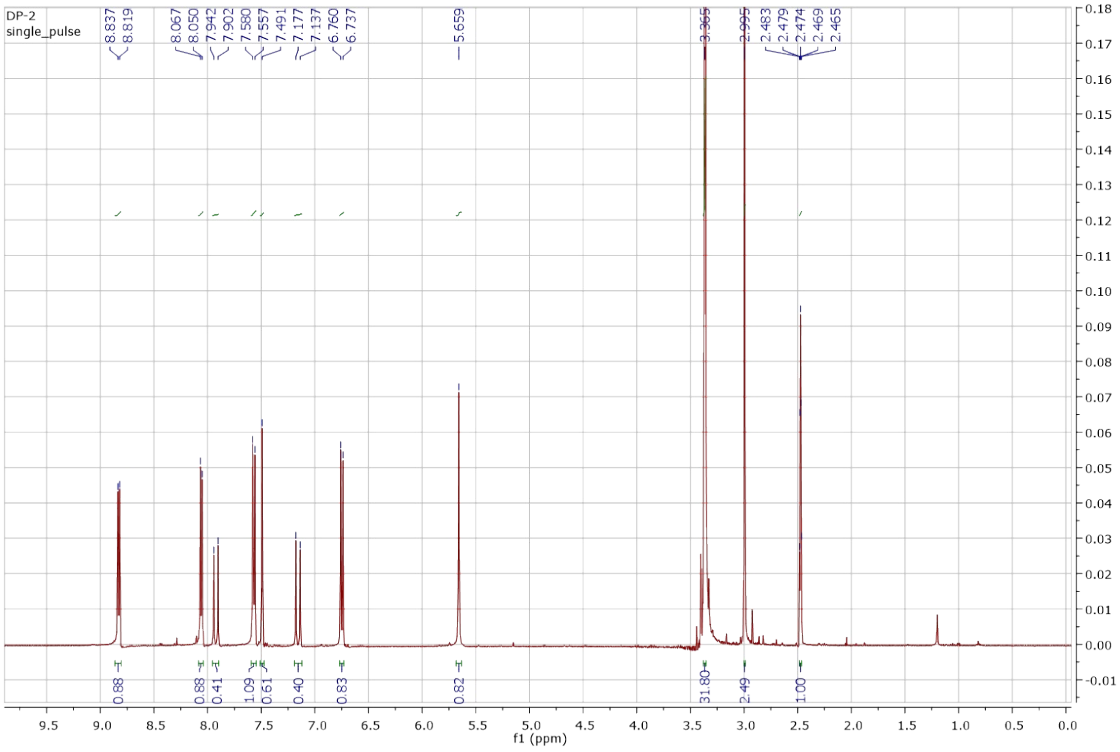

Figure: S4 ^{1}H NMR spectrum of **DPDM**

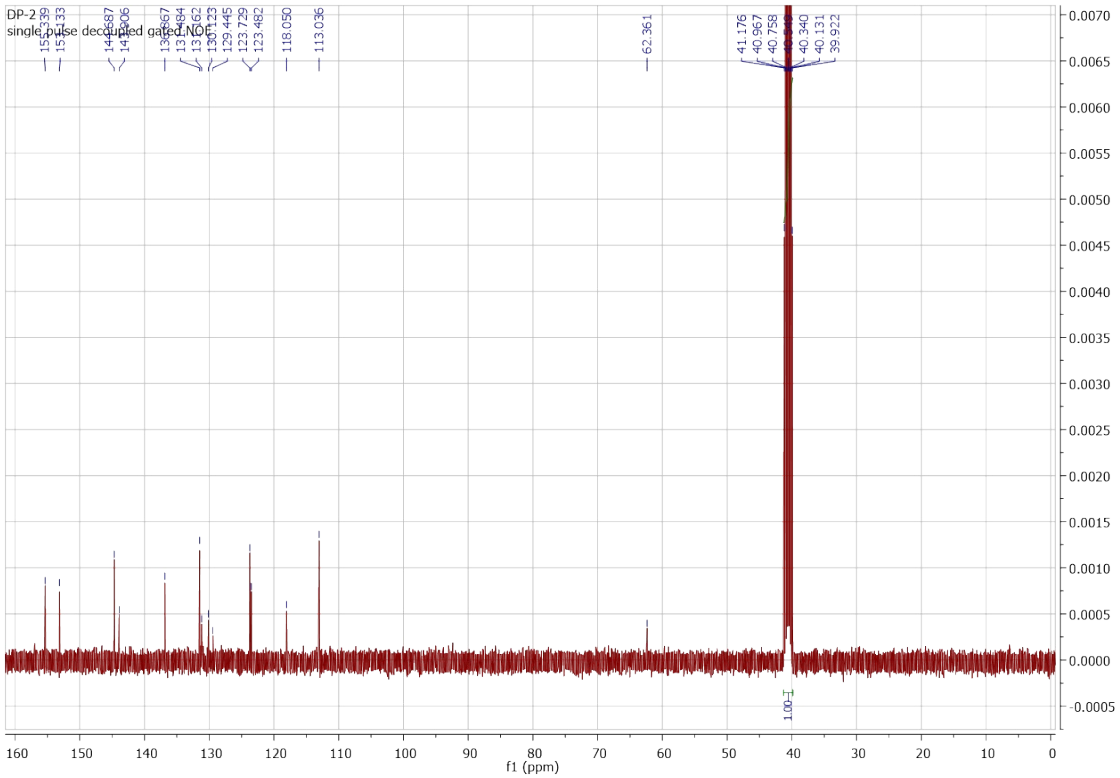

Figure: S5 ^{13}C NMR spectrum of **DPDM**

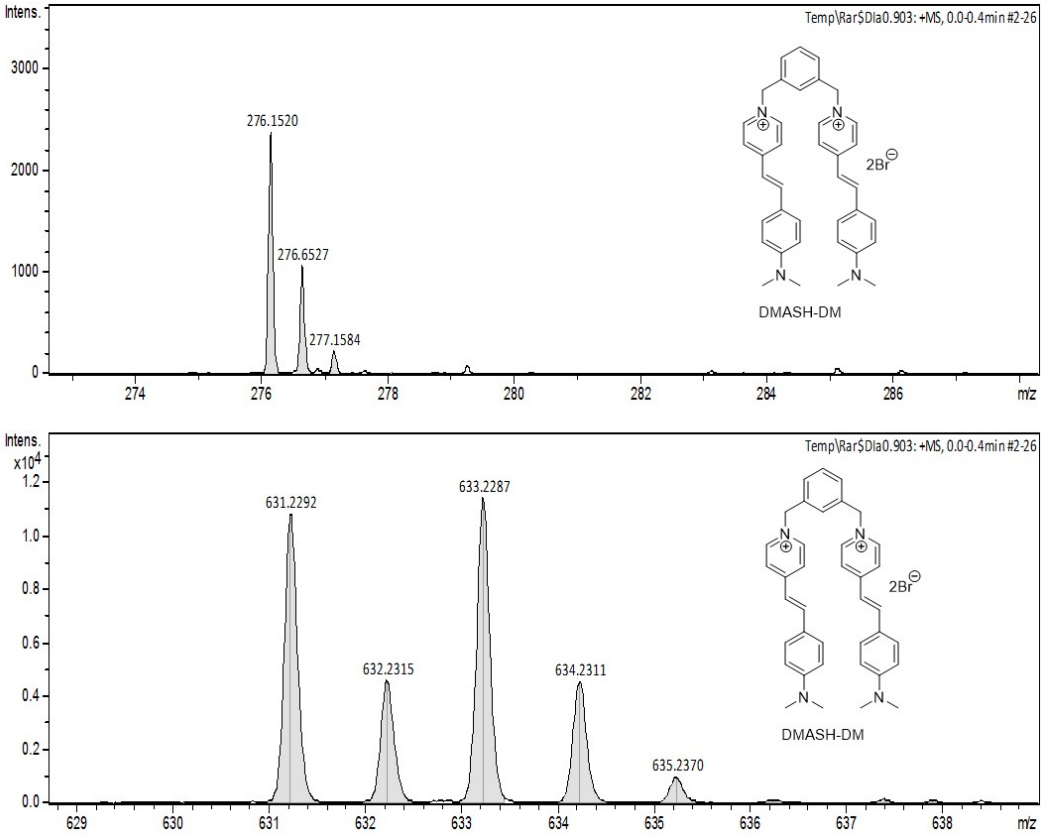

Figure: S6 HRMS spectrum of **DPDM**

Experimental Data

2.1. Materials

Chemicals and solvent of reagent grade were purchased from Aldrich, Spectrochem and used as it without any purifications. The common organic solvents including DMSO, Ethanol, and glycerol were used of HPLC grade. DMAS was synthesized by reaction between N-methyl pyridine and 4-(dimethylamino)-benzaldehyde as earlier synthesized in literature.The progress of reaction was monitored by silica gel coated TLC plate.

2.2. Instruments

The UV-visible experiments were performed on Shimadzu UV-2450 machine using slit width of 1.0 nm and glass quartz cell with 1 cm path length, at 25.0 ± 0.1^{o}C for all measurements. The fluorescence studies were performed by Horiba Fluorolog-3 with glass quartz cuvette of 1 cm path length. DLS experiments were performed at 25.0 ± 0.1^{o}C by using light scattering apparatus zeta sizer Nano ZS Malvern instrument LTD, UK. The deionized water was obtained from the ultra UV/UF Rions lab water system ultra 370 series which were used for preparing all solutions. ^{1}H and ^{13}C NMR spectra was recorded on JEOL 400MHz NMR machine using DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal reference. The chemical shifts are in ppm relative to TMS and coupling constant J in Hz, multiplicity (s= singlet, d= doublet, t=triplet, m= multiplets). High Resolution mass spectrum were recorded on Bruker Micro Toff/ QII (Germany). Circular dichroism (CD) studies were performed between 270 nm and 200 nm spectral range with continuous scanning rate (1 nm/min) on a JASCO, J-1500 circular dichroism spectrophotometer.

2.3. Optical analysis

The UV-Vis and fluorescence emission spectra of **DPDM** and **MPMB** were recorded in presence of various amounts of SAs at 25 ± 1^{o}C. The spectra were stored as ASCII files and then processed using Microsoft excel programme.

2.4. Anisotropy measurements

The fluorescence anisotropy experiments were performed by using excitation wavelength 490 nm and the emission wavelength 610 nm. During the anisotropy measurements, after each addition of BSA or HSA to the solution of fluorophore, the solution was well mixed by stirring for 2 minutes and was kept as it is for 2-3 minutes without disturbing to ensure the formation of stable complex between probe and BSA/HSA. Each anisotropy value was recorded as an average of ten measurements.

2.5. Fourier transform infrared (FT-IR) spectral analysis

The FTIR studies were performed to appraise the interactions of probes with BSA / HSA (500 μM) in water. The solutions of (i) BSA (500 μM), (ii) BSA (500 μM) + probe (100 μM); (iii) BSA (500 μM) + probe (250 μM), (iv) probe (100 μM), (v) probe (250 μM) in buffer were prepared separately. The FTIR spectra with 128 scans were recoded for each solution. A baseline correction was made in the IR spectra of BSA and its solutions with probe by subtracting the IR spectra of solvent (water) from (i) and the IR spectrum of solution of probe iv from (ii) and of probe v from solution (iii). For each probe, the same protocol was followed. All FTIR spectra were recorded in the spectral region of 4000 to 400 cm^{-1} by putting 10 μl of each solution on ATR probe. The de-convolution of amide I peak between 1600-1700 cm^{-1} was performed using origin 9.0 software which resulted in 5 peaks, and the area under each peak was then evaluated against the total area to find the percentage of each component α-helix, random, coil and β-sheet in secondary structure of BSA.

2.6. Fluorescence quenching of BSA / HSA

The fluorescence quenching experiment was performed to check the binding nature of BSA / HSA with probes. On excitation at 290 nm, the solution of BSA / HSA (10 μM) in HEPES buffer gave the fluorescence emission in 310-400 nm region which corresponds to tryptophan (Trp 213 and Trp 134 of BSA and Trp 214 of HSA) residues.

2.7. DLS studies

The stock and analyte solutions were filtered through 0.02 μM filter membrane to remove suspended impurities. The solutions of fluorescent probes (5 μM) with different concentrations of BSA were kept for 2 h to attain homogeneity of aggregates / complexes. Each solution (~ 1 ml) was placed in glass cuvette and was kept undisturbed at 25^{o}C for 2 minutes before recording the DLS spectra at least five times. The Zetasizer software was used for analysis of these DLS spectra.

2.8. Site marker displacement studies

The site marker displacement experiments were performed to know binding location of the probes in SAs. Warfarin – a selective binding drug at subdomain IIA (Sudlow's site I); ibuprofen - a selective binding drug at subdomain IIIA (Sudlow's site II) and bilirubin – a selective drug for subdomain IB were used as site markers. The solution of probes and SA

was gradually treated with aliquots of marker drug and after addition of each aliquot the fluorescence spectrum was recorded.

2.9. Time-resolved fluorescence analysis

Fluorescence lifetime studies of probes **MPMB** (5μM) and **DPDM** (5 μM) were performed in absence and presence of BSA / HSA. The concentrations of BSA 10 -100 μM (1 to 10 equivalents) were added to the solutions of **MPMB** and **DPDM** in HEPES buffer. Pulse excitation of 444 nm and emission between 520-700 nm was measured. The time resolved photoluminescence curves were fitted with mono and bi-exponential species and the average lifetime was calculated.

2.10. Density functional theory calculations

The ground state geometry optimizations of **MPMB** and **DPDM** were performed using density functional theory (DFT) with wb97xd/6-31G (d) level basis sets for structural optimization.

2.11. Molecular Docking studies

In order to scan the geometrically and energetically stable conformations of the probes **MPMB** and **DPDM**, molecular docking of these probes was in microenvironment of BSA. The crystal structure of BSA (pdb id: 4f5s) was retrieved from Protein Data Bank. The three-dimensional (3D) structures of the ligands were drawn using MOE and energy optimized using the AM1 semi-empirical approach with an RMS gradient of 0.001 kcalmol-1. The DPDM was stabilized in Z-conformation which attained nearly U-conformation during docking process. Before docking, the suitable binding sites for inserting the ligands in the active sites of BSA were determined by site finder, and a total of 28 binding sites in BSA were discovered for both the probes in the process. Finally, the ligands were docked to the favourable site of BSA determined by MOE's site finder using the Alfa PMI placement approach, and the best fitting domain was chosen for the docking research

2.13. Cell Culture and Treatment

2.13.1. Procurement and maintenance of cell line

Hela cell line was obtained from the National Centre for Cell Science (NCCS, Pune, India). These cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and antibiotic-antimycotic solution. The

Hela cells were cultured in cell culture flasks and incubated at humidified atmosphere at 37 ℃ under 5% CO_2 and 95% air mixture.

2.13.2 MTT assay

The cytotoxic potential of test sample towards Hela cell line was determined using MTT assay. The HeLa cells were cultured at the density of $8x10^3$ cells/well in a 96 well microplates in 100µL of DMEM medium and incubated for 24 hours. After that cells were treated with various concentrations of the test sample for 12 hours then added 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye solution in each well and incubation was continued for an additional 2 h for measuring the ability of viable cells to reduce it into purple coloured formazan. Subsequently, the media was removed from the wells with MTT solution and then added 100 µl of dimethyl sulphoxide (DMSO) to dissolve the intracellular insoluble purple-coloured formazan. Then absorbance was taken at 570 nm using a multi-well plate reader (BioTek Synergy HT). The growth inhibition % was calculated by the formula:

% Growth inhibition = A_0-$A_1/A_0 \times 100$

Where A_0 is the absorbance of control, and A_1 is the absorbance of the test sample.

2.13.3 Optical Microscopy

The cells were cultured at a density of 1.5×10^4 cells/well onto 24-well plate with 12mm coverslips. After reaching 70-80 % confluency and adherence the cells were treated with test sample. For co-localization experiments, HeLa cells were incubated **DPDM** then add the commercial Mitochondria Green Tracker for 30 min at 37 ˚C. HeLa Cells was treated with **DPDM** for 30 min and further incubated with BSA/HSA for 30 min at 37 ˚C. After treatment, the cells were washed two- three times with 1xPBS and coverslips having cells were mounted on the glass slides containing a drop of anti-fading reagent (Fluoromount; Sigma). The pictures were observed under a Nikon A1R Laser Scanning Confocal Microscope system (Nikon Corporation, Tokyo, Japan).

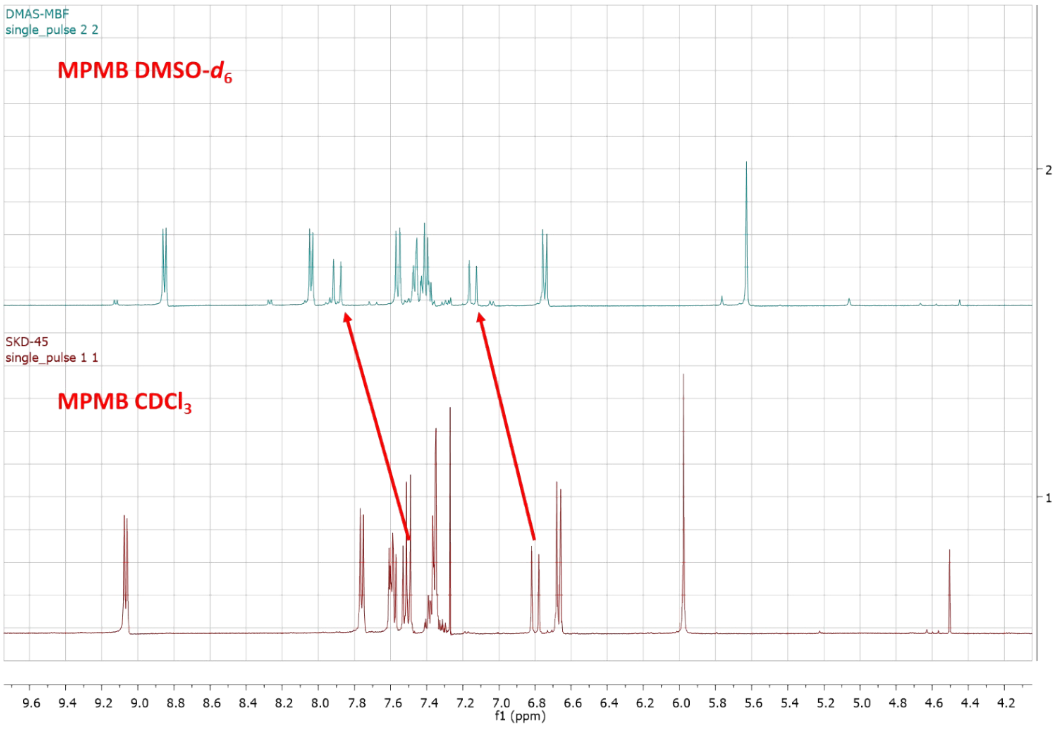

Figure S7. ^{1}H NMR spectrum of **MPMB** in $CDCl_3$ and DMSO-d_6

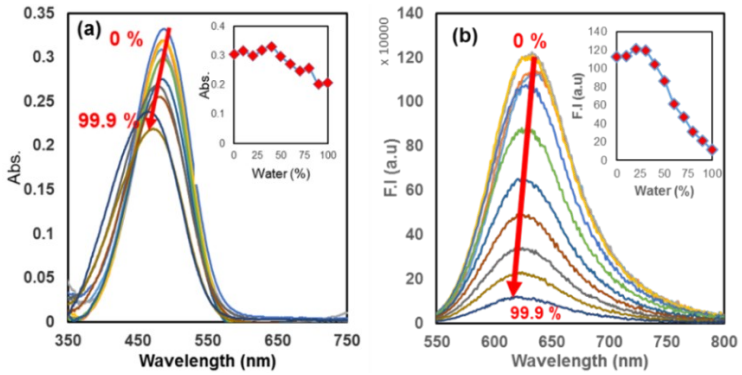

Figure S8. Change in (A) absorption and (B) fluorescence spectra of **MPMB** in DMSO-H_2O (HEPES buffer, pH 7.4) binary mixtures with increasing water fraction.

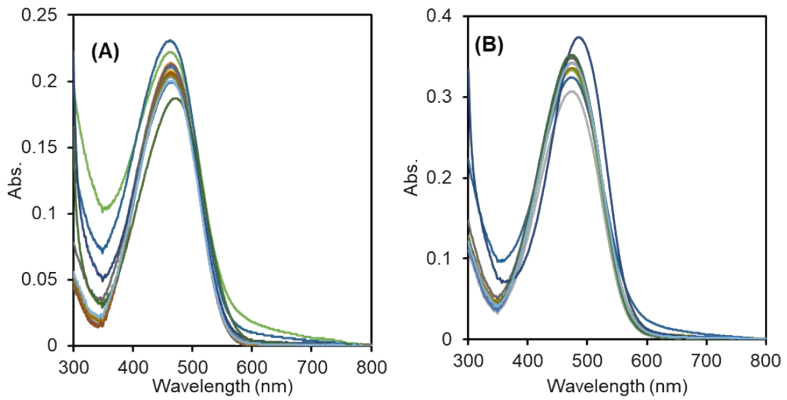

Figure S9. Change in absorbance spectrum of (A) **MPMB** and (B) **DPDM** in presence of various protein and biomolecules

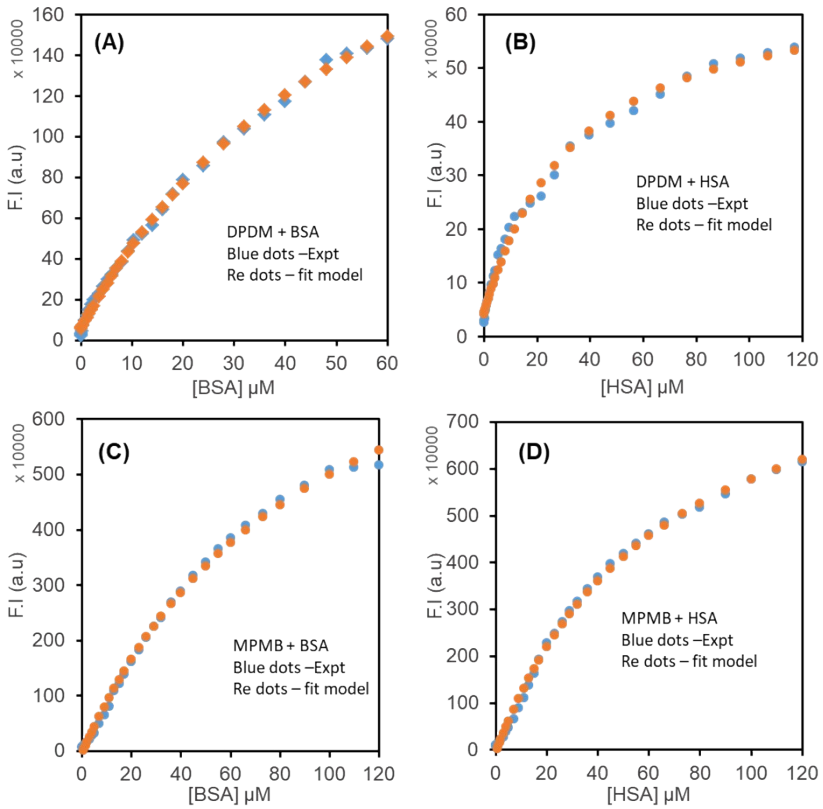

Figure S10. Plot of graph of (A and B) **DPDM** and (C and D) **MPMB** against different concentrations of BSA/HSA and its fit model

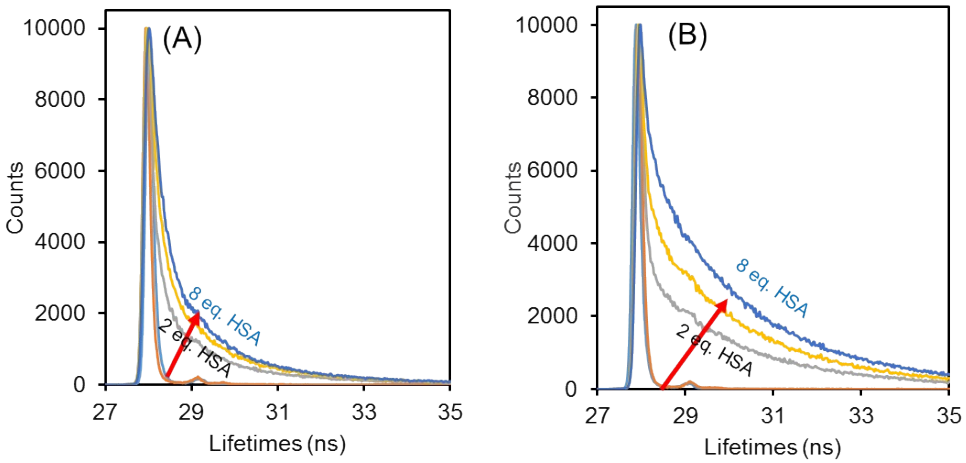

Figure S11. Change in lifetime spectrum of (A) DPDM and (B) MPMB in presence of HSA.

Table S1. Effect of BSA/HSA on the fluorescence life time of **DPDM.**

S.No	code	T1 (%) ns	T2 (%) ns	X2 (chi-square)
1	DPDM	0.01 (97.44)	0.21 (2.56)	1.22
2	DPDM + 2EQ BSA	0.37 (33.02)	2.14 (66.98)	1.35
3	DPDM + 4EQ BSA	0.84 (33.96)	2.47 (66.04)	1.03
4	DPDM + 2EQ HSA	0.22 (17.59)	1.63 (82.41)	1.34
5	DPDM + 4EQ HSA	0.42 (23.14)	1.98 (76.86)	1.33

Table S2. Effect of BSA/HSA on the fluorescence lifetime of **MPMB**

S.No	code	T1 (%) ns	T2 (%) ns	X2 (chi-square)
1	MPMB	0.03 (49.95)	0.3 (50)	1.38
2	MPMB + 2EQ BSA	0.72 (18.36)	2.57 (81.64)	1.15
3	MPMB + 4EQ BSA	0.84 (19.32)	2.63 (80.68)	1.19
4	MPMB + 2EQ HSA	0.81 (18.45)	2.76 (81.55)	1.23
5	MPMB + 4EQ HSA	0.85 (17.27)	2.77 (82.73)	1.27

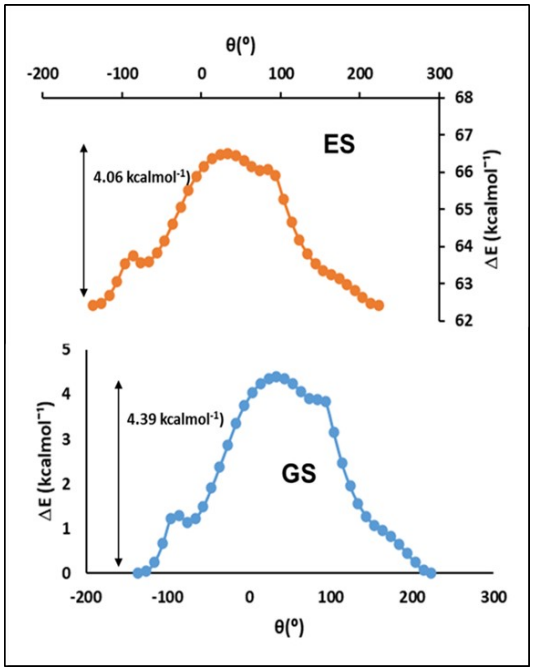

Figure S12. Potential energy curve for the dihedral angle for DPDM

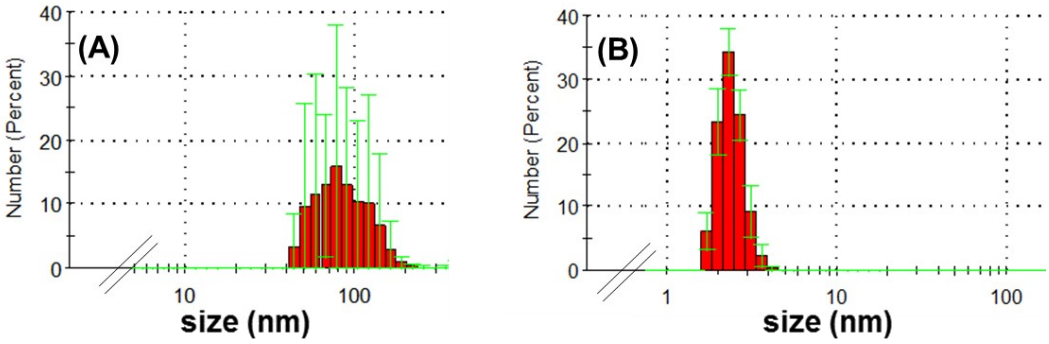

Figure S13. DLS graph of (A) **MPMB**; (B) MPMB + BSA in HEPES buffer containing (0.1 % DMSO)

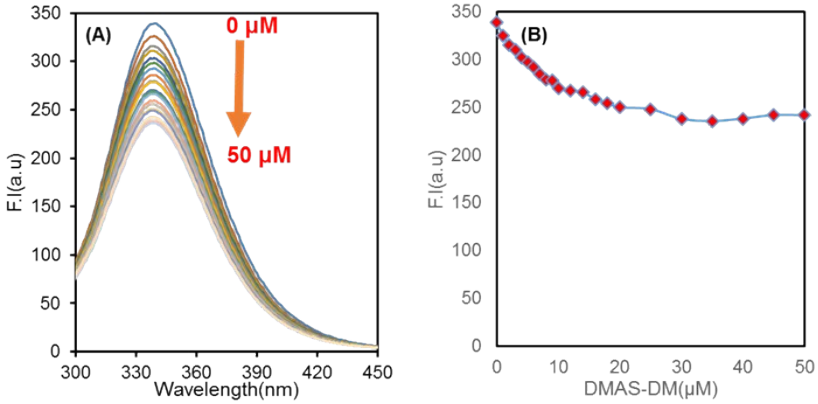

Figure S14. Change in the (A) fluorescence spectrum and (B) fluorescence intensity of BSA (10 μM) with addition of **DPDM** in HEPES buffer.

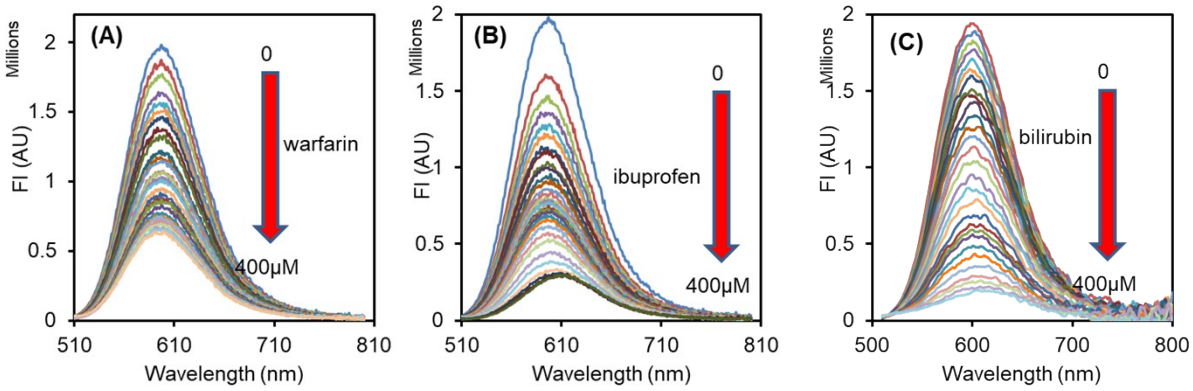

Figure S15. Change in fluorescence spectrum of **DPDM∩BSA** with addition of (A) warfarin (B) ibuprofen and (C) bilirubin

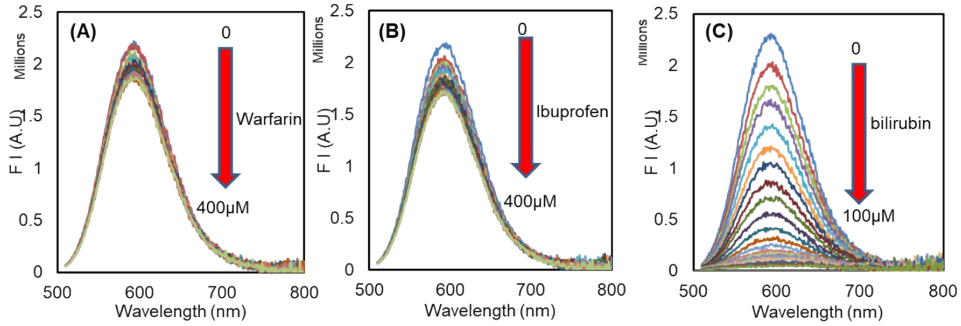

Figure S16. Change in fluorescence spectrum of MPMB∩BSA with addition of (A) warfarin (B) ibuprofen and (C) bilirubin

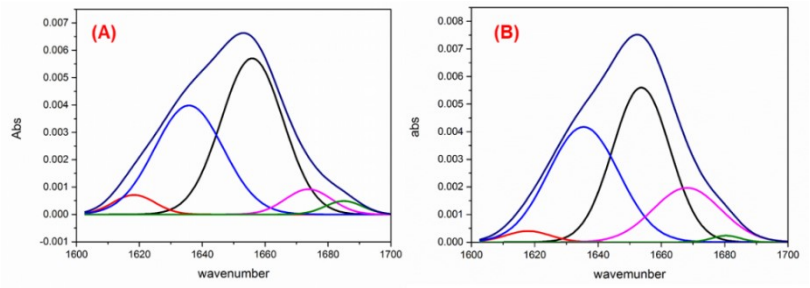

Figure S17. Second derivative resolution enhancement and curve-fitted amide I region (1700-1600 cm^{-1}): (A) free BSA (500 μM) and (B) its complex with DPDM (100 μM)

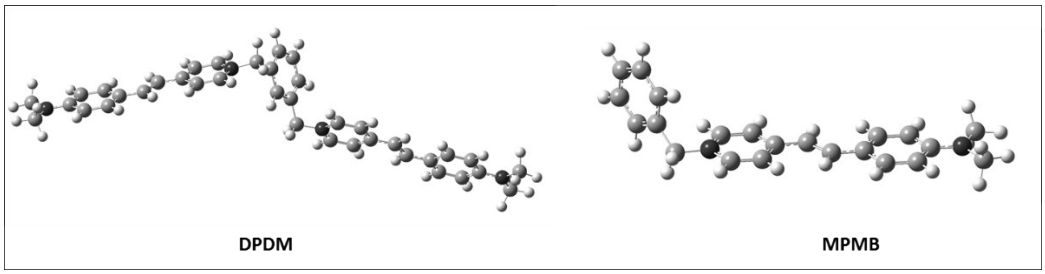

Figure S18. Gaussian 9.0 optimized structures of **DPDM** and **MPMB**.

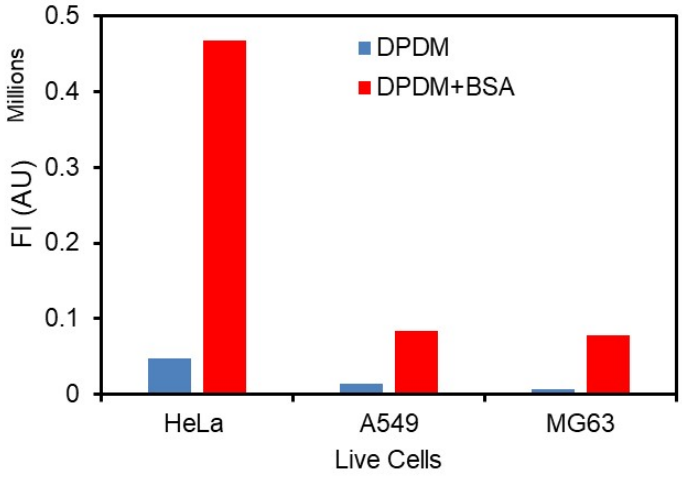

Figure S19. The relative fluorescence intensity of DPDM before and after treatment with BSA in HeLa, A549 and MG63 cells