Red-emissive tripodal nanoprobe for discrimination of serum albumin with conformational change from ʏ- to ω-like and probing mitochondrial viscosity

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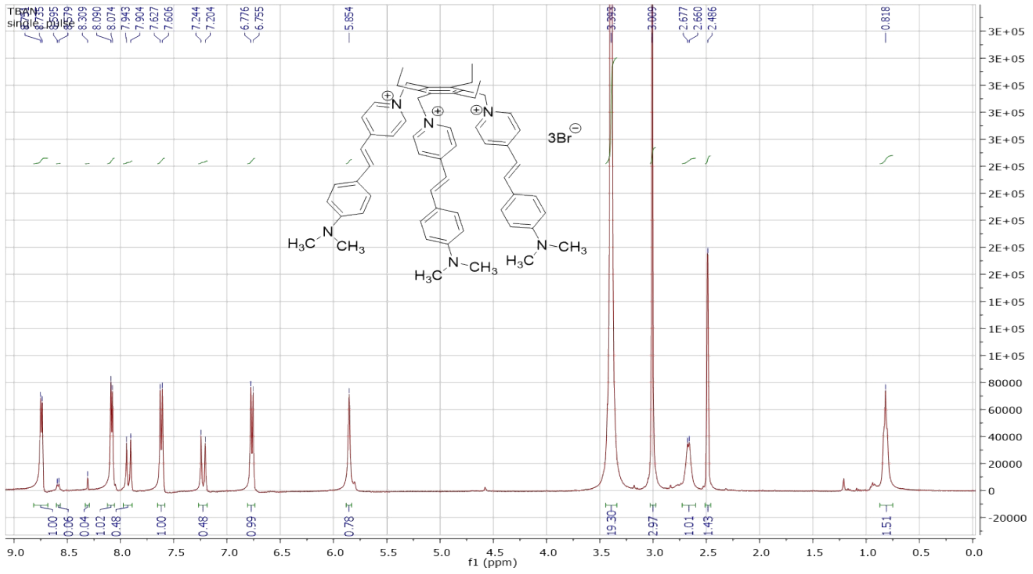

Figure S1: ^{1}H NMR Spectrum of **DMAS-TP** (400 MHz, DMSO-d_6)

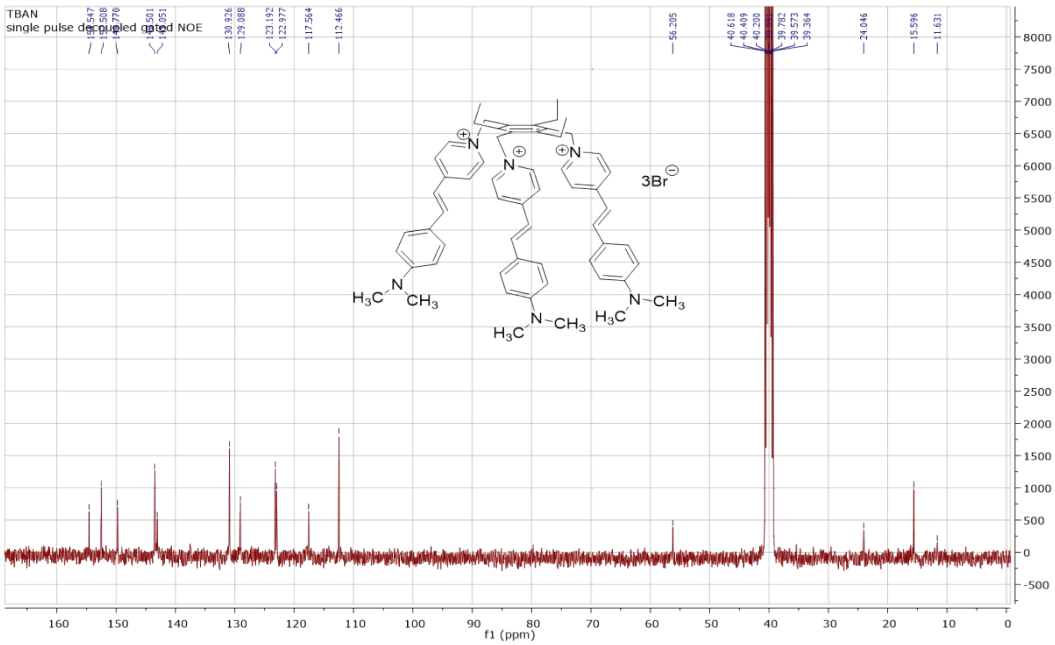

Figure S2: ^{13}C NMR Spectrum of **DMAS-TP** (100 MHz, DMSO-d_6)

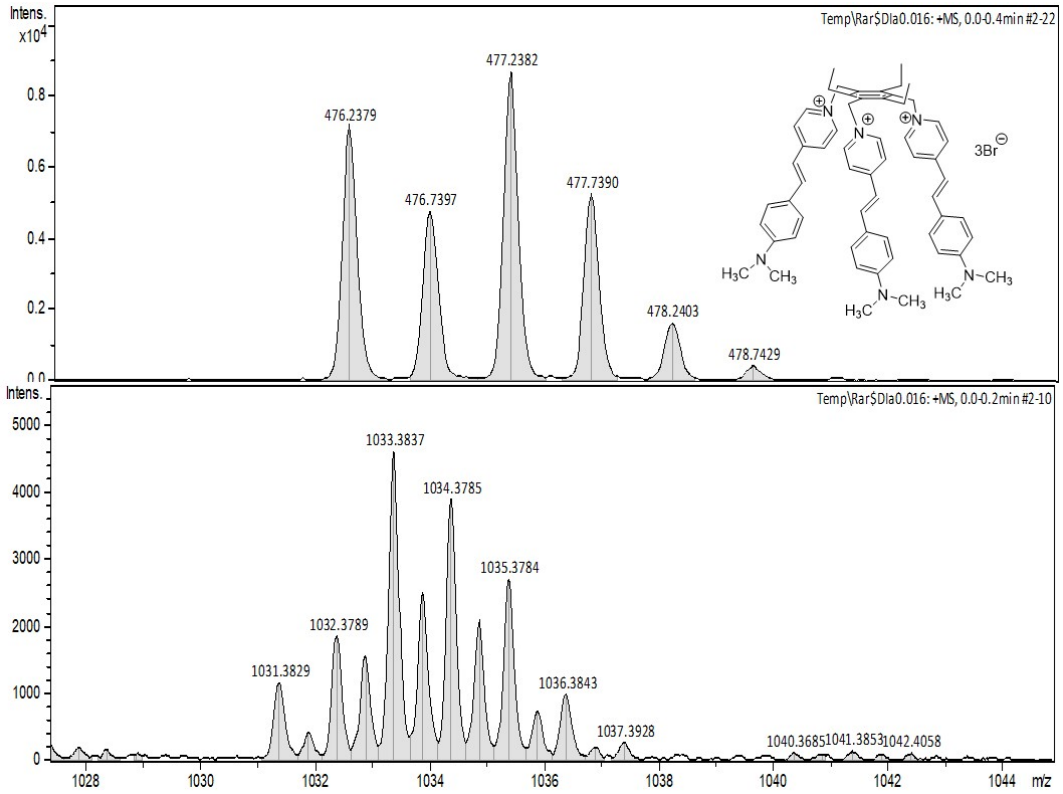

Figure S3: HRMS spectrum of **DMAS-TP** indicating the fragments at $C_{60}H_{69}BrN_6^{2+}$, $C_{60}H_{69}N_6Br^{2+}$.

2. Experimental data

2.1. Materials

Analytical-grade chemicals and solvents were purchased from Aldrich and Spectrochem and used without further purification. Common organic solvents, including DMSO, CH_3CN, and diethyl ether were used in HPLC grade. The compound DMAS was synthesized through a condensation reaction between N-methyl pyridine and 4-(dimethylamino)-benzaldehyde, following a method previously described in the literature. The progress of the reaction was monitored using a silica gel-coated TLC plate.

2.2. Methods and apparatus

^1H and ^{13}C NMR spectra were recorded on a JEOL 400MHz NMR instruments machine using DMSO-d_6 as solvent and tetramethylsilane (TMS) as an internal standard reference. The peak in Chemical shift in ppm relative to TMS and coupling constant *J* in Hz, multiplicity (s= singlet, d= doublet, t=triplet, m= multiplets). HRMS (High-Resolution mass spectrum) was recorded on Bruker Micro Toff/ QII (Germany). The UV-visible studies were performed on the Shimadzu UV-2450 machine using a slit width of 1.0 nm and a glass quartz cell with a 1 cm path length, thermostat stated at 25.0 ± 0.1°C for all measurements. The fluorescence studies were performed using Horiba Fluorolog-3 with

a glass quartz cuvette of 1 cm path length. Circular dichroism (CD) studies were performed between 300 nm to 200 nm spectral range on a JASCO, J-1500 circular dichroism spectrophotometer. DLS experiments were performed at 25.0 ± 0.1°C by using zeta sizer Nano ZS Malvern instrument LTD, UK. HR-TEM images were obtained with a JEOL JEM-2100 electron microscope operating at an acceleration voltage of 200 kV. The deionized water was obtained from the ultra UV/UF Rions lab water system ultra 370 series which was used for preparing all solutions.

2.3 UV-vis and fluorescence studies

The stock solution of fluorescent tripodal **DMAS-TP** (1 mM) was prepared in DMSO solvent and further diluted as required. For the recording of UV-Vis and fluorescence spectrum, stock solutions (100 μL) were taken in a 10 ml volumetric flask and diluted with HEPES buffer (0.01 M, pH 7.2) for performing experiments. The freshly prepared standard stock solution of various biomolecules and proteins like BSA, HSA, cysteine, homocysteine, gulthonine, histidine, pepsin, trypsin, aspartic acid, bromelain, lysine, Haemoglobin, pyrophosphates in deionized millipore water.

2.4 Detection Limit

The limit of detection (LOD) was calculated from fluorescence studies. To determine the signal-to-noise (S/N) ratio, the emission of **DMAS-TP** was measured three times to calculate the standard deviation. The LOD was determined using the equations as per IUPAC guidelines.

$$LOD = 3\sigma/m$$

Where σ = standard deviation of blank measurements, m is the slope of intensity versus concentration of sample

2.5 Quantum Yield Calculations

The quantum yield (Φs) of solutions of **DMAS-TP** was measured with an integrated sphere using excitation wavelength at 490 nm.

2.6 Binding constants

To rationalize the formation of various stoichiometric complexes and binding constants, the whole spectral data of the titration experiments was evaluated using the software SPECFIT-32 program. The program performs global analysis of the equilibrium and kinetic systems with a singular value of decomposition and nonlinear regression modeling by the Levenberg-Marquardt method. The program simulates the fluorescence data

obtained experimentally. The stoichiometry of the species formed, the distribution of species, and their association constants are determined through the fit model.

2.7 Anisotropy measurement

The fluorescence anisotropy experiments were performed using an excitation wavelength of 490 nm and an emission wavelength of 620 nm. During the anisotropy measurements, after each addition of BSA/HSA to the solution of the **DMAS-TP**, the solution was well mixed by stirring for 2 min and kept as it was for 2-3 min without disturbing to ensure the formation of a stable complex between **DMAS-TP** and BSA/HSA. Each anisotropy value was recorded as an average of ten measurements.

2.8. Time-resolved fluorescence analysis

Fluorescence lifetime studies of tripodal nanoprobe **DMAS-TP** were performed in the absence and presence of BSA / HSA. The concentrations of BSA/HSA 10 -100 μM (1 to 10 equivalents) were added to the solutions of **DMAS-TP**. 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.

2.9. Site marker displacement studies

The site marker displacement experiments were conducted to identify the binding locations of the probes in serum albumin (SA). Warfarin (warf), a selective binding drug for subdomain IIA (Sudlow's site I); ibuprofen (IB), a selective binding drug for subdomain IIIA (Sudlow's site II); and bilirubin (BB), a selective binding drug for subdomain IB, were used as site markers. The complex of **DMAS-TP** and BSA was gradually treated with aliquots of the site marker drugs, and after each addition, the fluorescence spectrum was recorded.

2.10. Molecular Docking Studies

Molecular docking studies were carried out to gain a better understanding of the specific binding site of the **DMAS-TP** in the protein microenvironment, as well as to investigate the binding behavior of the ligands with BSA. The three-dimensional (3D) structures of **DMAS-TP** were drawn using MOE and energy-optimized using the AM1 semi-empirical approach with an RMS gradient of 0.001 kcal mol^{-1}. The Protein Data Bank was used to download the crystal structure of BSA (**pdb id: 4f5s**). Before docking, the suitable binding sites for inserting the ligands in the active sites of BSA were determined by the site finder, and 28 binding sites in BSA were discovered in the process.

2.11. DLS studies

The stock solutions of **DMAS-TP** and analytes were filtered through a 0.02 μM filter membrane to remove suspended impurities. The **DMAS-TP** (5 μM) solutions with BSA were allowed to sit for 2 h to ensure the formation of homogeneous aggregates/ complexes. Approximately 1 ml of each solution was placed in a glass cuvette and left undisturbed at 25 °C for 2 min before recording the DLS spectra at least five times. The Zetasizer software was then used to analyze these DLS spectra.

2.12. SEM sample preparations

The solutions of **DMAS-TP** and **DMAS-TP∩BSA** were filtered through a 0.02 μM filter membrane to remove interfering impurities. Each of these solutions (10 μL) was put on the pre-cleaned separate glass slides and placed in the thermostat at 25 ^{o}C for 24 h. During this period the films were dried.

2.13. TEM sample preparations

The solutions of **DMAS-TP** and **DMAS-TP∩BSA** were filtered through a 0.02 μM filter membrane to remove interfering impurities. Each of these solutions (2 μl) was placed on separate copper grids and allowed to dry in the thermostat at 25 ^{o}C.

2.14. Cell Culture and Treatment

2.14.1. Procurement and maintenance of cell line

The HeLa cell line was obtained from the National Centre for Cell Science (NCCS, Pune, India). The HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and an antibiotic-antimycotic solution. The HeLa cells were cultured in cell culture flasks and incubated in a humidified atmosphere at 37 °C with a 5% CO_2 and 95% air mixture.

2.14.2 MTT assay

The cytotoxic potential of the test sample towards the HeLa cell line was determined using the MTT assay. HeLa cells were cultured at a density of 8×10^3 cells per well in 96-well microplates with 100 μL of DMEM medium and incubated for 24 h. Subsequently, the cells were treated with various concentrations of the test sample for 12 h. After this treatment, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye solution was added to each well, and incubation continued for an additional 2 h to allow viable cells to reduce the MTT into purple-colored formazan. The media containing the MTT solution was then removed, and 100 μL of dimethyl sulfoxide was added to each well to dissolve the intracellular, insoluble purple-colored formazan.

Absorbance was measured 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 × 100

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

2.14.3 Optical Microscopy

The cells were cultured at a density of 1.5 × 10^4 cells per well in a 24-well plate with 12 mm coverslips. After reaching 70-80% confluency and adherence, the HeLa cells were treated with the sample. For viscosity experiments, HeLa cells were incubated with nystatin followed by **DMAS-TP** for 30 min at 37 °C. For co-localization experiments, HeLa cells were incubated with **DMAS-TP** for 30 min, then treated with commercial Mitochondria green Tracker for 30 min at 37 °C. After treatment, the cells were washed two to three times with 1x PBS, and the coverslips with cells were mounted on glass slides containing a drop of anti-fading reagent (Fluoromount; Sigma). The images were observed using a Nikon A1R Laser Scanning Confocal Microscope system (Nikon Corporation, Tokyo, Japan).

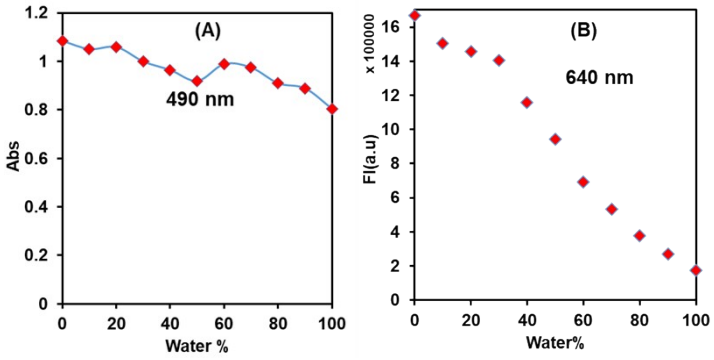

Figure S4. Change in absorption (A) and Emission (B) intensity of **DMAS-TP** with increasing the fraction of water

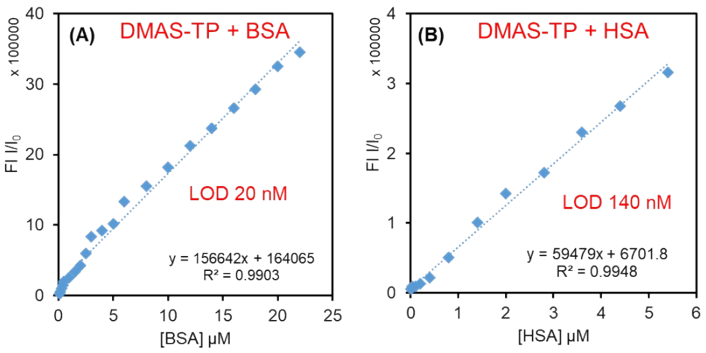

Figure S5. The plot FI *vs* the concentration of BSA (A) and HSA (B) for determination of the limit of detection.

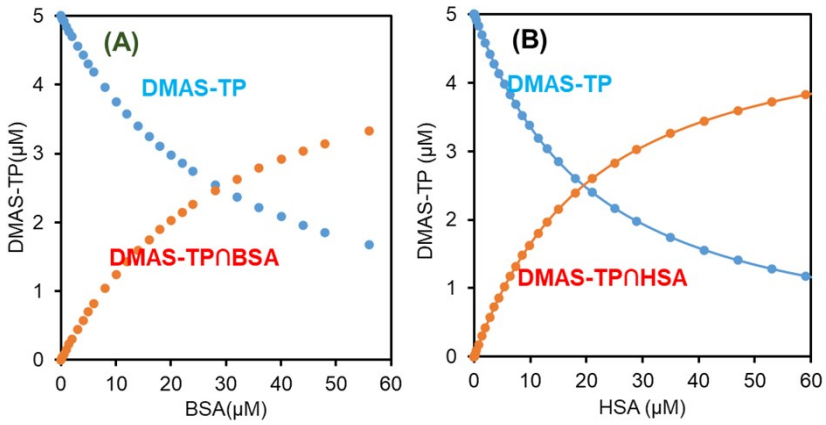

Figure S6. The plot of graphs of **DMAS-TP** against different concentrations of BSA (A) and HSA (B) and its fit model

Table S1 – Effect of BSA/HSA on the fluorescence lifetime of **DMAS-TP**

S. No	code	T1 (%) ns	T2 (%) ns	X^2 (chi-square)
1	DMAS-TP	0.01 (97.6)	0.23 (2.4)	1.05
2	DMAS-TP+ 2EQ BSA	0.28 (19.58)	1.61 (80.42)	1.30
3	DMAS-TP + 4EQ BSA	0.46 (26.72)	2.02 (73.28)	1.29
4	DMAS-TP + 8EQ BSA	0.48 (23.75)	2.09 (76.25)	1.30
5	DMAS-TP + 2EQ HSA	0.28 (29.79)	1.35 (70.21)	1.16
6	DMAS-TP + 4EQ HSA	0.42 (33.99)	1.87 (66.01)	1.23
7	DMAS-TP + 8EQ HSA	0.45 (34.54)	1.79 (65.46)	1.14

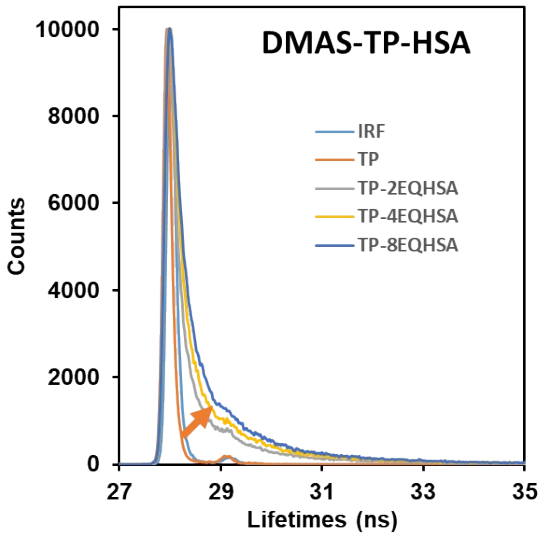

Figure S7. The change in the lifetime spectrum of tripodal **DMAS-TP** with increasing concentration of HSA.

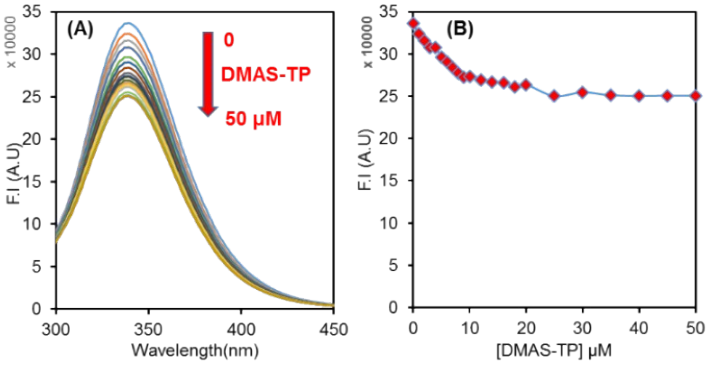

Figure S8. Change in the fluorescence spectrum of BSA (10 µM) upon excitation at 290 nm with the addition of an increasing amount of tripodal **DMAS-TP.**

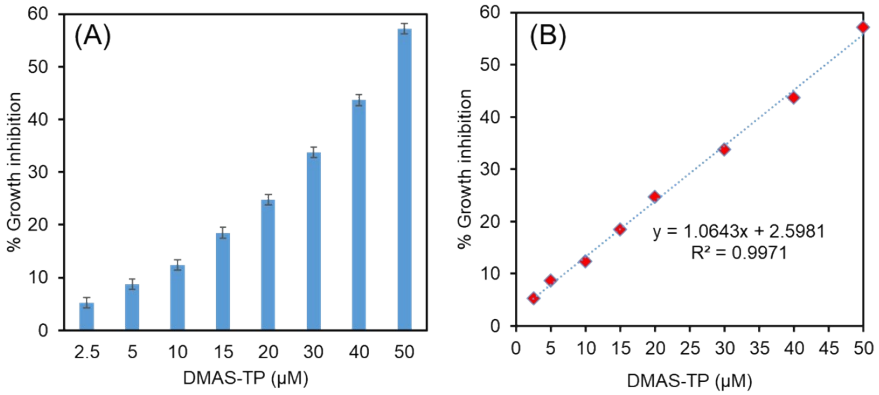

Figure S9. MTT assay of **DMAS-TP**.

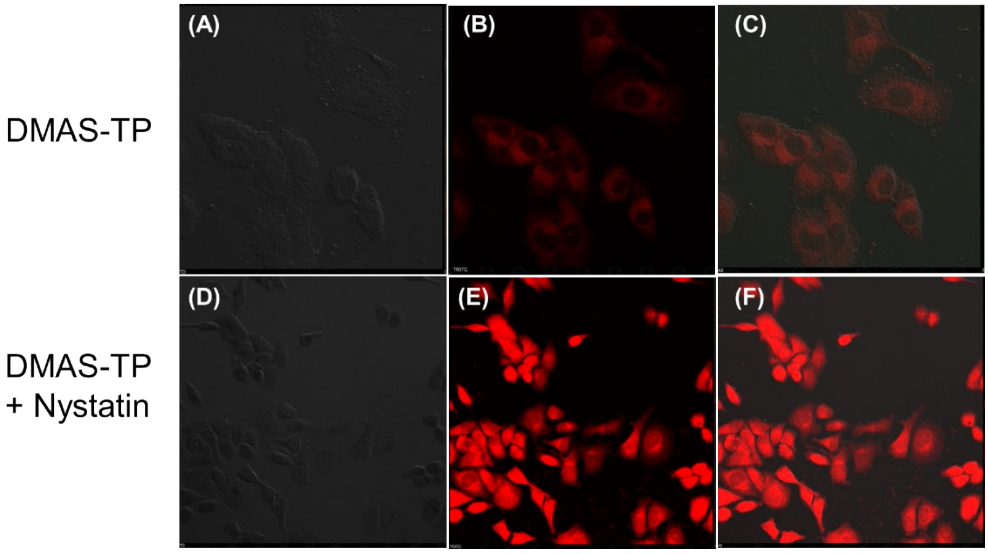

Figure S10. Confocal imaging of viscosity in HeLa cells incubated with **DMAS-TP** (5 µM) for 30 min (A) bright field; (B) fluorescence in red channel; (C) merged image of A and B. The incubation of HeLa cells with nystatin and after with **DMAS-TP** (5 µM) for 30 min, (D) bright-field; (E) fluorescence in red channel; (F) merged image of D and E**.**