Electronic supplementary information

Red-NIR emissive probe for selective detection of albumin in urine samples and live cells†

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I. Experimental details

1.1 Materials and methods

All reagents and solvents were purchased from Sigma Aldrich and used without any further purification unless mentioned. All the air and moisture sensitive reactions were carried out under argon or N_2 atmosphere. All the proteins and enzymes (pepsin, trypsin, peroxidase, glucose oxidase, γ-globulin, BSA and HSA) were purchased from Sigma Aldrich. Absorption and fluorescence spectra were recorded with Agilent Cary series UV-Vis-NIR absorption and Agilent Cary eclipse fluorescence spectrophotometers, respectively. Polyacrylamide gel electrophoresis (PAGE) was performed using Biorad apparatus and the gels were imaged in Biorad Chemidoc MP imaging system. Data obtained was plotted and analysed in origin 8.5 or prism 5. All the experiments were performed in 10 mM PBS buffer with pH 7.4. For absorption and fluorescence studies, sample volume was fixed to 700 μM and 500 μM, respectively. Probe (**TG-SA)** concentration of 5 μM was used in most of the experiments unless mentioned.

1.2 Synthetic of probe TG-SA

1-(2-Hydroxyphenyl)butane-1,3-dione (**1**). To a solution of 1-(2-hydroxyphenyl)ethanone (1 g, 8.35 mmol) dissolved in 20 mL ethyl acetate, sodium (0.9 g, 38.57 mmol) was added under N_2 condition. Then the reaction mixture was stirred for 4 h to obtain a greyish solid product, which was filtered and dissolved in 100 mL deionized water. The pH of the solution was was adjusted to neutral and the solution was extracted with ethyl acetate (EtOAc), dried over Na_2SO_4, filtered, and concentrated to yield the product **1** as a brown sticky solid, which was used in the next reaction without any further purification.

2-Methyl-4H-chromen-4-one (**2**). Compound was **1** (0.7 g, 3.9 mmol) dissolved in a mixture of acetic acid (7 mL) and sulfuric acid (0.5 mL), and refluxed at 120 °C for about 45 min. Then the reaction mixture was poured into ice cold water and pH was adjusted to neutral using saturated Na_2CO_3 solution. The aqueous solution was extracted with dichloromethane (DCM), dried over anhydrous Na_2SO_4, filtered, and concentrated to obtain the product **2**, which was directly used in the next reaction without any further purification.

2-(2-Methyl-4H-chromen-4-ylidene)malononitrile (**3**). To a solution of acetic anhydride (15 mL) **2** (2.4 g, 15.5 mmol) and malononitrile (1.3 g, 21.1 mmol) were added and refluxed at 140 °C for 14 h. The reaction mixture was concentrated and distilled water (30 mL) was added, followed by reflux for 1 h. Then the reaction mixture was extraction with DCM, dried over anhydrous Na_2SO_4, filtered, and concentrated. The obtained crude product was purified by column chromatography (Hex/Et_2O: 8/2) to yield compound **3** as reddish orange solid (Yield, 40%).

^{1}H NMR (*$CDCl_3$*, 400 MHz) δ 8.93-8.90 (m, 1H), 7.74-7.69 (m, 1H), 7.47-7.43 (m, 2H), 6.72 (s, 1H), 2.44 (s, 3H).

^{1}H NMR (*DMSO-d_6*, 400 MHz) δ 8.68 (s, 1H), 7.91-7.86 (m, 1H), 7.68 (d, J = 5.3 Hz, 1H), 7.62-7.58 (m, 1H), 6.77 (s, 1H); ^{13}C NMR (*DMSO-d_6*, 100 MHz) δ 164.3, 153.2, 152.4, 135.3, 126.2, 124.5, 118.9, 116.6, 115.5, 104.8, 59.5, 19.9; HRMS (ESI-MS): found. 161.0597 $[M+H]^+$, calcd. 160.0524 for $C_{10}H_8O_2$.

(E)-2-(2-(4-(Diethylamino)-2-hydroxystyryl)-4H-chromen-4-ylidene)malononitrile (**TG-SA**). To the solution of **3** (100 mg, 0.48 mmol) and 4-(diethylamino)-2-hydroxybenzaldehyde (105 mg, 0.57 mmol) dissolved in toluene (15 mL), piperidine (0.23 mL) and acetic acid (0.23 mL) were added under argon atmosphere, and refluxed at 115 °C for 3 h. The reaction mixture was

concentrated and the crude product was purified by column chromatography (Hex/Et_2O: 8/2) to obtain **TG-SA** as a crystalline green solid (Yield 34%). ^{1}H NMR (*DMSO-d_6*, 400 MHz) δ 10.09 (s, 1H), 8.71 (d, J = 8 Hz, 1H), 7.94-7.83 (m, 2H), 7.70-7.75 (m, 1H), 7.58-7.53 (m, 1H), 7.58-7.53 (m, 2H), 7.04-7.02 (m, 2H), 6.78 (s, 1H), 6.32-6.29 (m, 1H), 6.32-6.29 (m, 1H), 6.13 (d, J = 2 Hz, 1H), 3.37 (t, J = 5.2 Hz, 4H), 1.13 (t, J = 7.2 Hz, 6H); ^{13}C NMR (*DMSO-d_6*, 100 MHz) δ 160.5, 159.1, 152.2, 152, 135.9, 134.7, 130.1, 125.7, 124.4, 118.7, 118.1, 117.3, 116.7, 110.8, 110.2, 104.8, 104, 97; HRMS (ESI-MS): found. 384.1703 $[M+H]^+$, calcd. 384.1712 for $C_{24}H_{22}N_3O_2$.

1.3 Calculation of molar excitation coefficient (ε): Molar excitation coefficient is a measure of how strongly a molecule attenuates or absorbs light at a given wavelength. It is an intrinsic property of the molecule. Absorbance for varying concentrations (1 μM, 2 μM, 3 μM, 5 μM, 8 μM and 10 μM) of **TG-SA** in DMSO, methanol and DCM was measured, then the absorbance; maxima were plotted against concentrations and linearly fitted using origin 8.5 software to obtain the ε for **TG-SA** (Fig. S1).

1.4 photostabilty: **TG-SA** (5 μM) was dissolved in PBS buffer [PBS,10 mM:DMSO (1:1)] and irradiated with 400W light source for 2 h. Then the probes integrity was analysed through absorption spectroscopy using beer lambert's law.

1.5 Determination of HSA in urine samples: Concentration dependent study of HSA in presence of **TG-SA** (5 μM) was performed to obtain a standard curve for the change in fluorescence intensity at 655 nm (λ_{ex}= 530 nm) with increase in HSA concentration. All the urine samples were obtained from diagnostic centre and 400 μL of each sample without any further dilution was incubated with **TG-SA** (5 μM) for 10 min. Then the fluorescence intensity from each sample was recorded at 655 nm and used to determine the HSA concentration from following equation (Fig. S2)

$$y = mx + c$$

Where y = fluorescence intensity of **TG-SA** at 655 nm, **m** is slope, **x** is concentration of HSA, and **c** is intercept on Y-axis

For determining the HSA concentration in urine clinically, dipstick based strips were used. The area of the detection on the strip is coated with an indicator and tetrabromophenol blue. At pH 3 the strip is yellow in colour in the absence of protein, whereas in the presence of albumin the strip colour changes. The colour change is compared with standard chart and the albumin concentration is determined.

1.6 Polyacrylamide gel electrophoresis (PAGE): PAGE experiments were performed using Biorad apparatus. Native PAGE: Initially, the HSA was diluted in buffer (30% glycerol and 0.05% bromophenol blue in 150 mM tris/HCl buffer). The samples were loaded directly into 10% polyacrylamide gel and run at 150 V for 30 min, followed by washing with distilled water (3-4 times). SDS-PAGE: Initially, the samples (HSA, γ-globulin, trypsin, pepsin, glucose oxidase, egg white) were diluted with reducing buffer (1.2% SDS, 30% glycerol and 0.05% bromophenol blue in 150 mM tris/HCl buffer). The samples were loaded directly into 10% polyacrylamide gel and run at 150 V for 30 min, followed by washing with distilled water (3-4 times).

TG-SA *staining*. The gel was transferred to fixing buffer (50% methanol, 10% acetic acid, 100 mM ammonium acetate) and incubated for 15 min with constant shaking, then washed with distilled water for 3-4 times. Subsequently, the gel was transfer to **TG-SA** (30 μM) solution and incubate for 10 min with constant shaking, then washed (4 times) and imaged in gel documentation system (Fig. S8).

Coomassie staining. The gel was transfer to fixing buffer (50% methanol, 10% acetic acid, 100 mM ammonium acetate) and incubated for 15 min with constant shaking, then washed with distilled water for 3-4 times. The gel was transferred to EZ blue solution (Coomassie blue stain, Sigma Aldrich) and incubated for 60 min with constant shaking, then washed (4 times) and allowed it to stand in distilled water for 24 h to obtain the clear bands.

1.7 Detection of HSA *in cellulo*. HeLa and PC12 cells were seeded in 12-well plates at 30,000 cells/well in DMEM (10% FBS, 1% PS) and RPMI (10% HS, 5% FBS and 1% PS) complete medium and incubated for 24 h. Cells were treated with **TG-SA** (5 μM) and Hoechst (300 nM) in serum-free DMEM or RPMI medium. After 20 min incubation, the cells were washed with PBS and fresh DMEM or RPMI complete media was added. Then the live cells were imaged under fluorescence microscope (Leica Dmi8) using DAPI and Rho filters for Hoechst and **TG-SA**, respectively. For studying SA degradation inside the live cells with time, HeLa cells were seeded in 35 mm petridish with 30,000 cells in DMEM (10% FBS, 1% PS) complete medium and incubated for 24 h. Then the cells were treated with **TG-SA** (5 μM) in serum-free DMEM medium and incubated for 20 min. The cells were washed with PBS and fresh serum free DMEM media was added. Then the live cells were imaged under fluorescence microscope (Leica Dmi8) at various time points (0, 1, 2, 3 and 5 h) and analysed through Leica LAX software.

2. Supporting figures

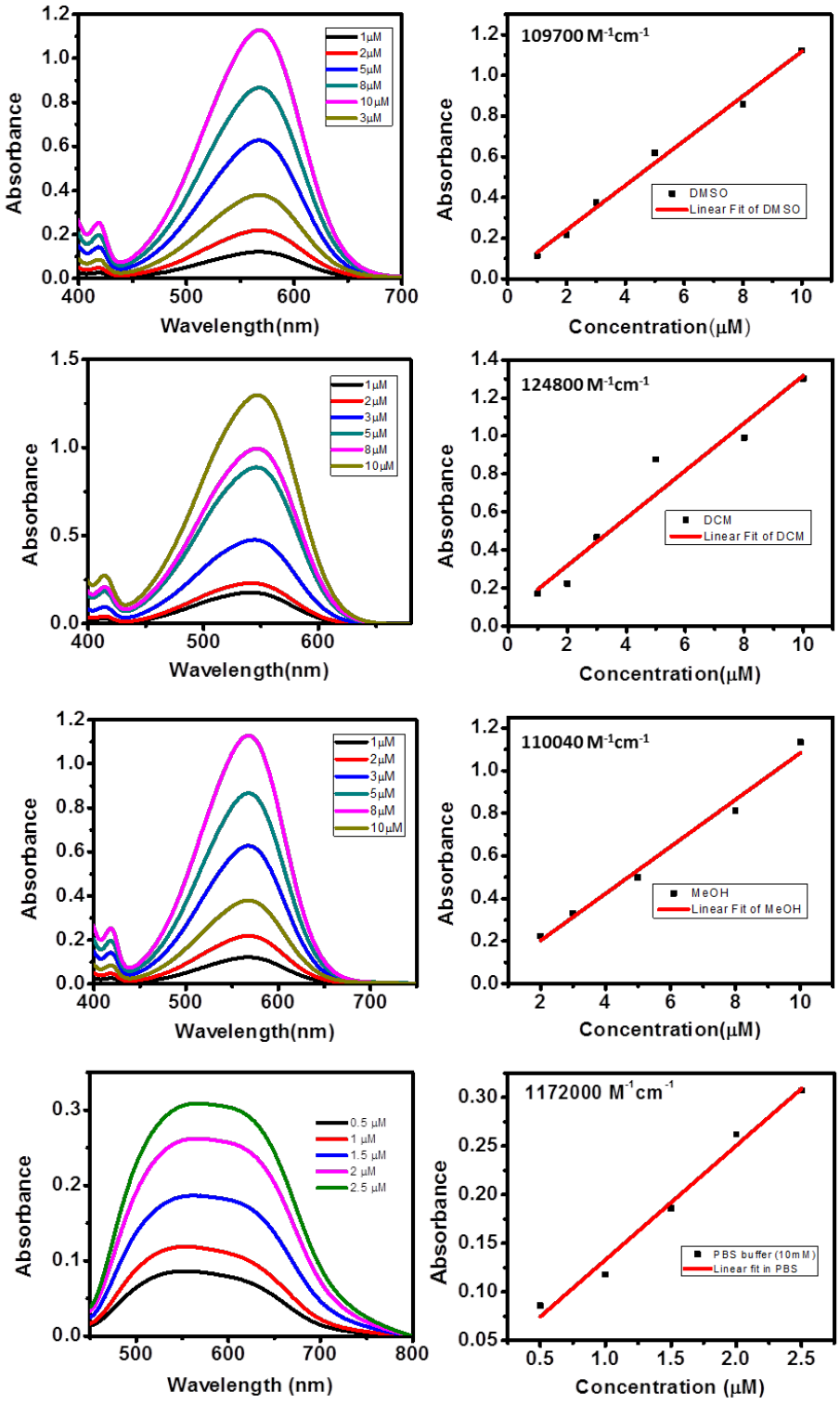


Fig. S1 Molar absorption coefficient of **TG-SA** in different solvent systems

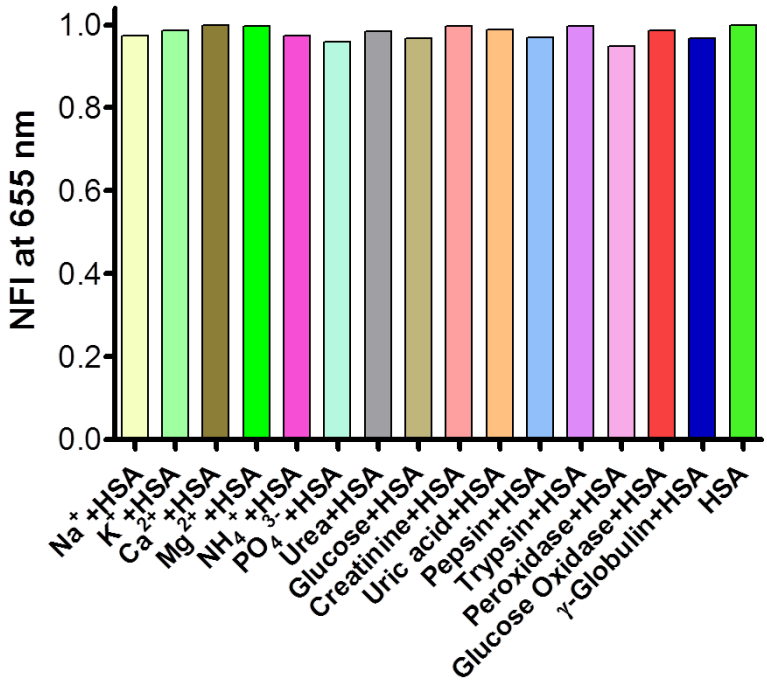


Fig. S2 Normalized fluorescence intensity (NFI, λ_{ex}= 530 nm) of **TG-SA** (5 μM) at 655 nm in presence of Na^+, K^+, Ca^{2+}, Mg^{2+}, NH_4^+, PO_4^{3-}, urea, glucose, creatinine, uric acid, pepsin, trypsin, peroxidase, glucose oxidase, γ-globulin with HSA (100 mg/L) protein.

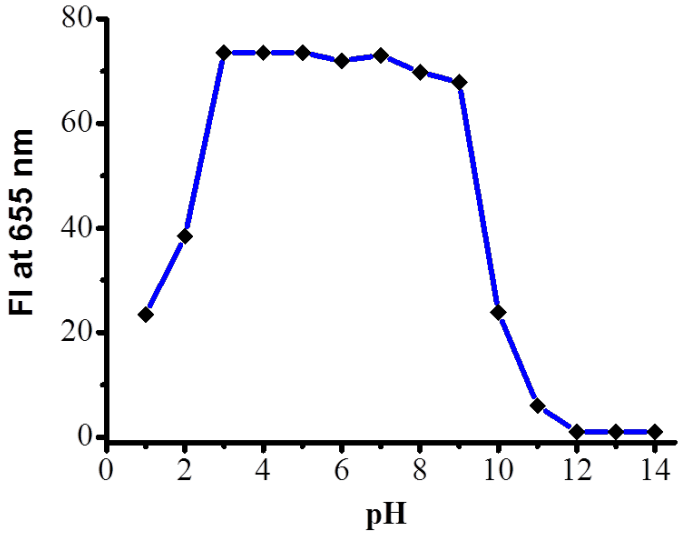


Fig. S3 Fluorescence intensity (FI) of **TG-SA** (5 mM) at 655 nm (λ_{ex}= 530 nm) recorded at different pH in PBS buffer [PBS,10 mM:DMSO (1:1)].

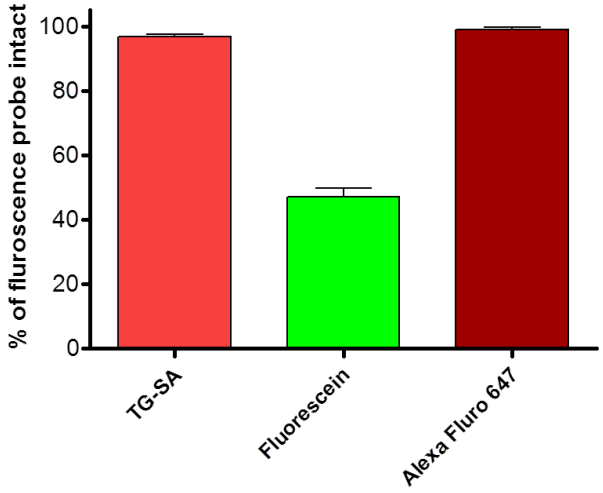


Fig. S4 Percentage of fluorescence probe intact (**TG-SA**, fluorescein and Alexa Flour 647) in PBS buffer after irradiating with 400W light source for 2 h.

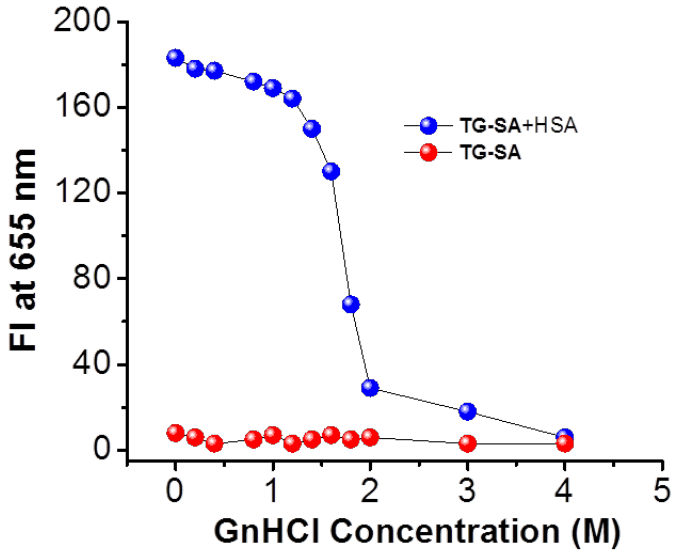


Fig. S5 Fluorescence intensity (FI) of **TG-SA** (5 µM) and **TG-SA**-HSA complex (5 µM of **TG-SA** + 100 mg/L HSA) monitored at 655 nm (λ_{ex}= 530 nm) upon addition of increasing concentration of GnHCl (0.2, 0.4, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2, 3 and 4 M).

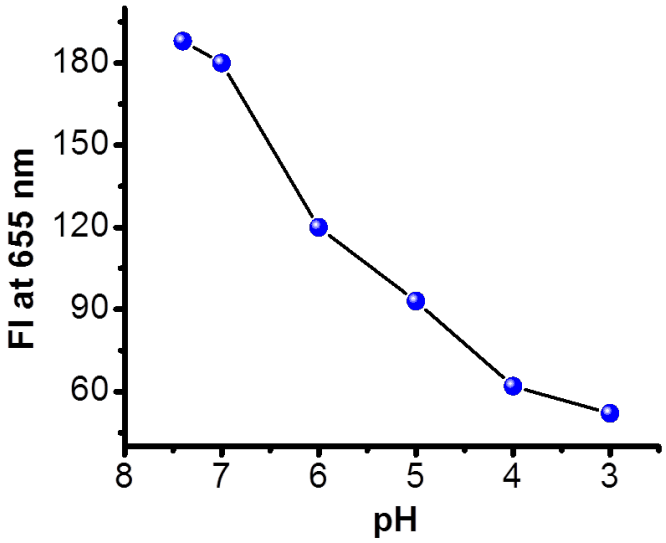


Fig. S6 Fluorescence intensity (FI) of **TG-SA-**HSA complex (5 µM of **TG-SA** + 100 mg/L HSA) at 655 nm (λ_{ex}= 530 nm) with decrease in pH.

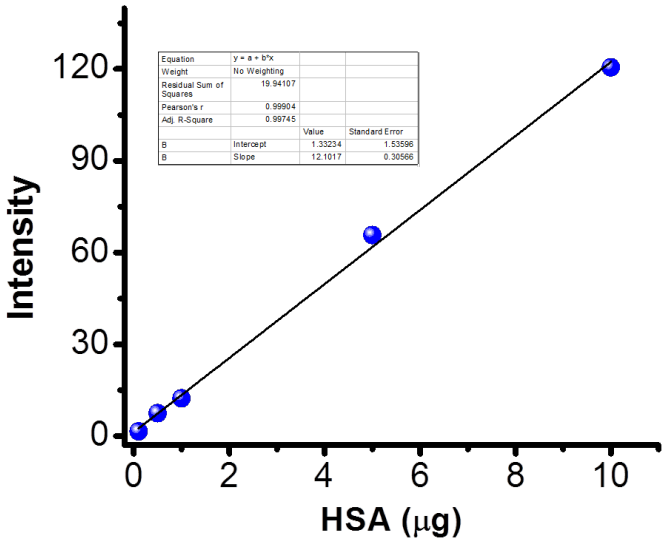


Fig. S7 Linear relationship between fluorescence intensity (green LED excitation) of **TG-SA** (30 µM) and HSA concentration (0.1, 0.5, 1, 5 and 10 µg) in PAGE (Fig b-i).

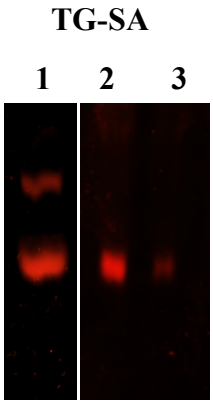


Fig. S8 Fluorescence imaging of polyacrylamide gel loaded with HSA protein (1), and egg white 5 μL (2), 1 μL (3) and stained with **TG-SA** (30 μM).

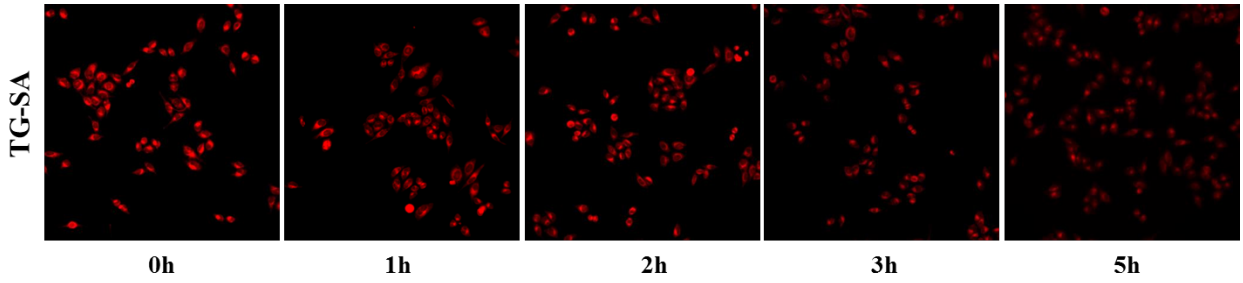
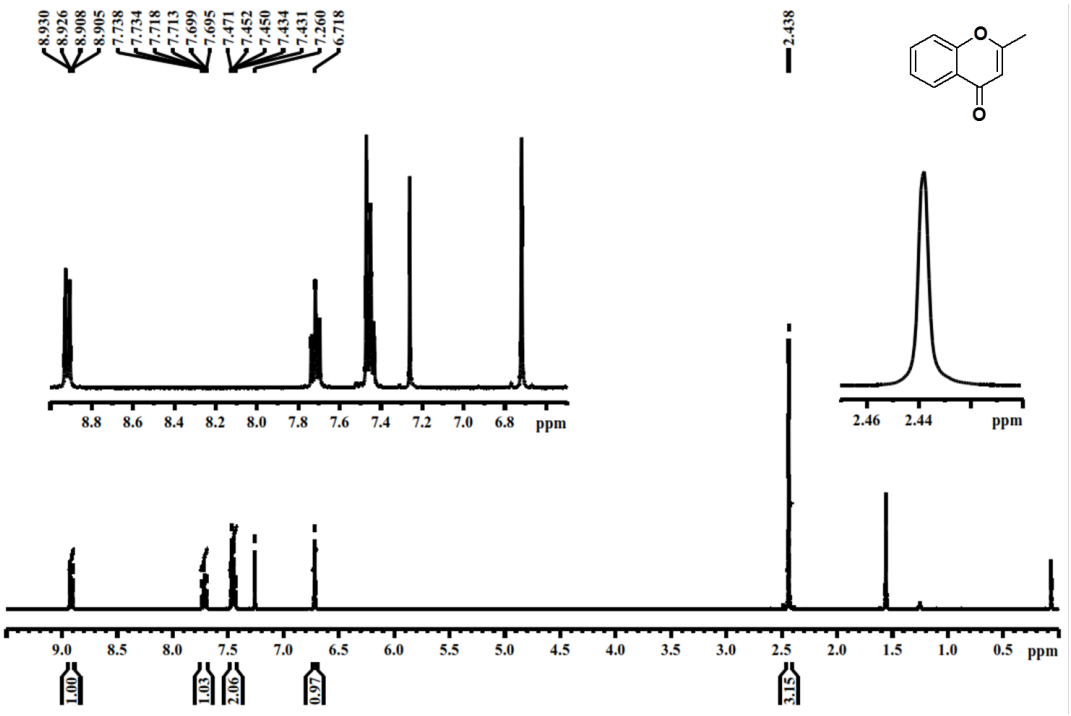


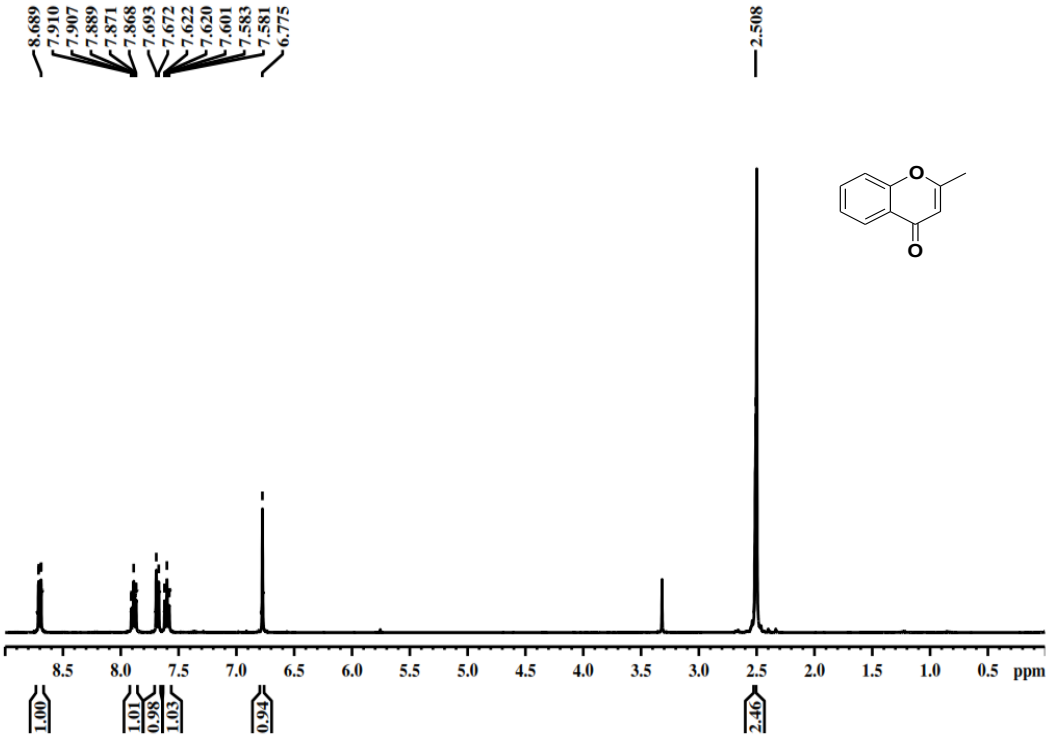
Fig. S9 Fluorescence microscopy images of Hela cells stained with **TG-SA** (2 μM) in serum free media (DMEM without FBS) and imaged at various time points (0, 1, 2, 3 and 5 h) to monitor cellular depletion of albumin.

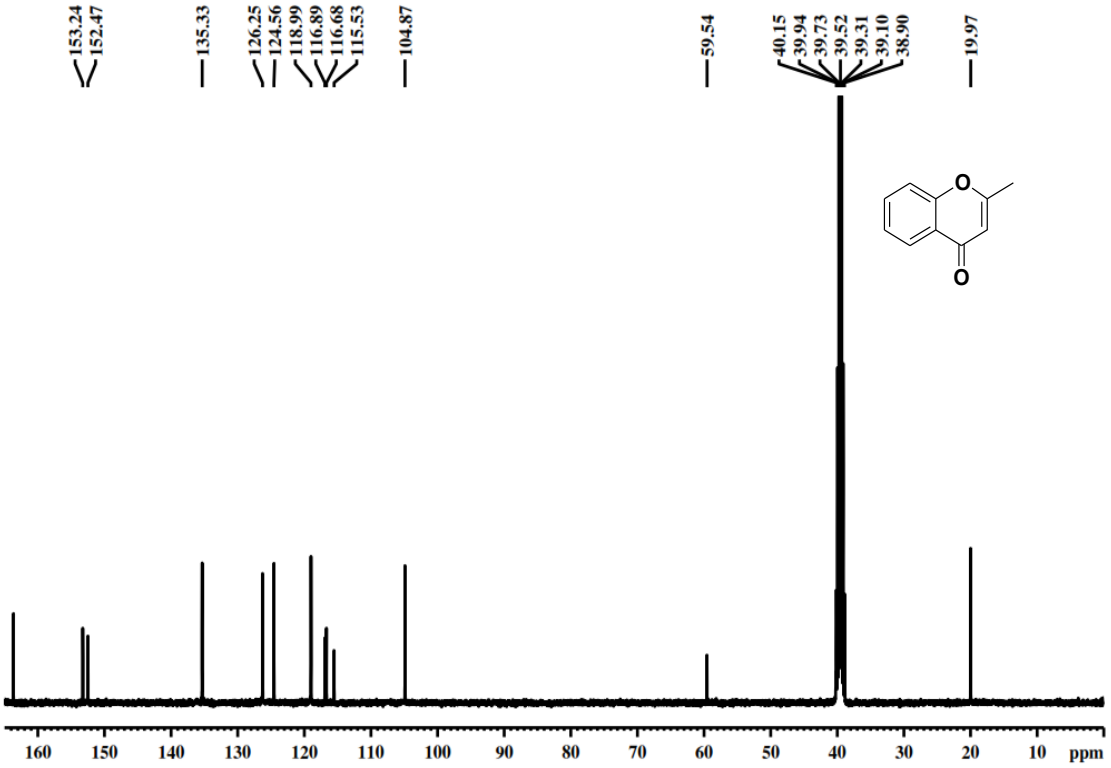
3. NMR Data

Compound 3 ^{1}H NMR in *CDCl$_3$*



Compound 3 ^{1}H NMR in *DMSO-d$_6$*

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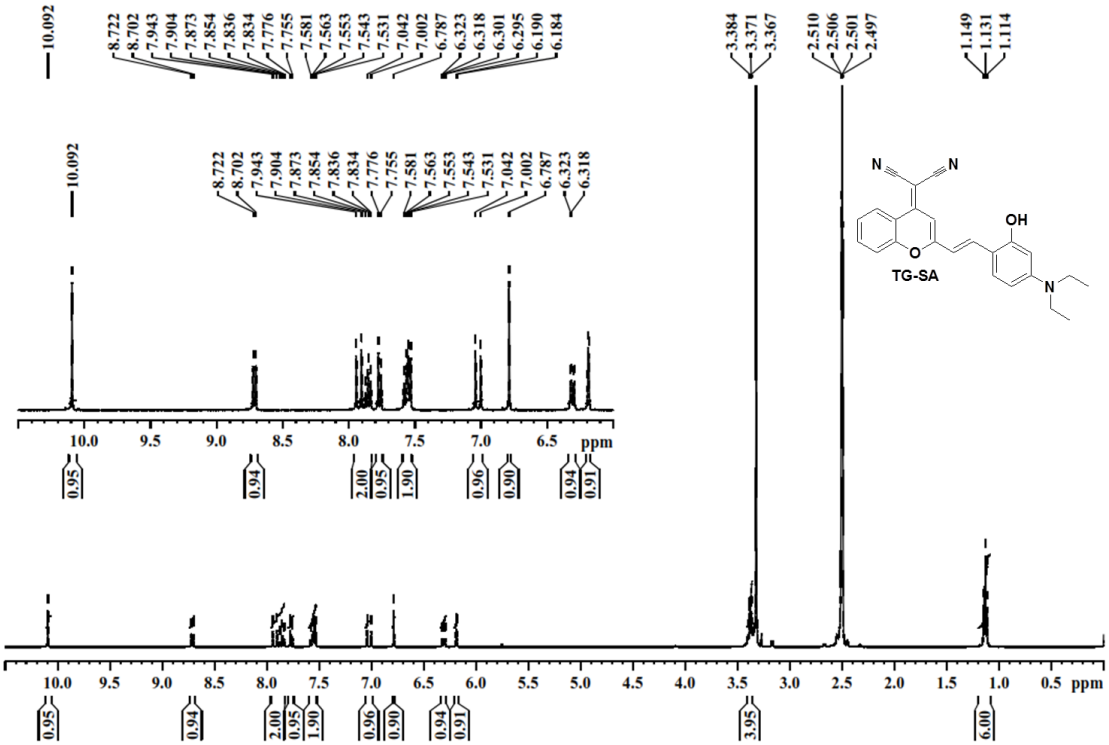
152.47
135.33
126.25
124.56
118.99
116.89
116.68
115.53
104.87
59.54
40.15
39.94
39.73
39.52
39.31
39.10
38.90
19.97
O
O
160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm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8.722
8.702
7.943
7.904
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7.581
7.563
7.553
7.543
7.531
7.042
7.002
6.787
6.323
6.318
6.301
6.295
6.190
6.184
3.384
3.371
3.367
2.510
2.506
2.501
2.497
1.149
1.131
1.114
N
N
OH
O
TG-SA
N
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 ppm
0.95 0.94 2.00 0.95 1.90 0.96 0.90 0.94 0.91
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
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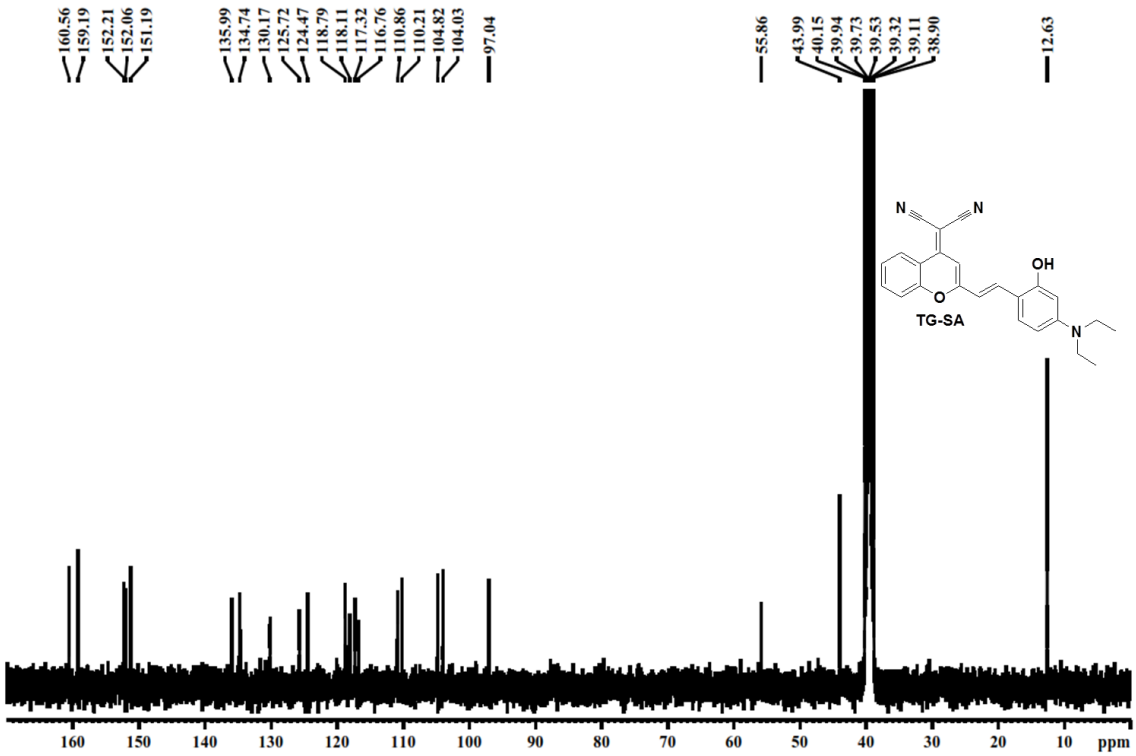
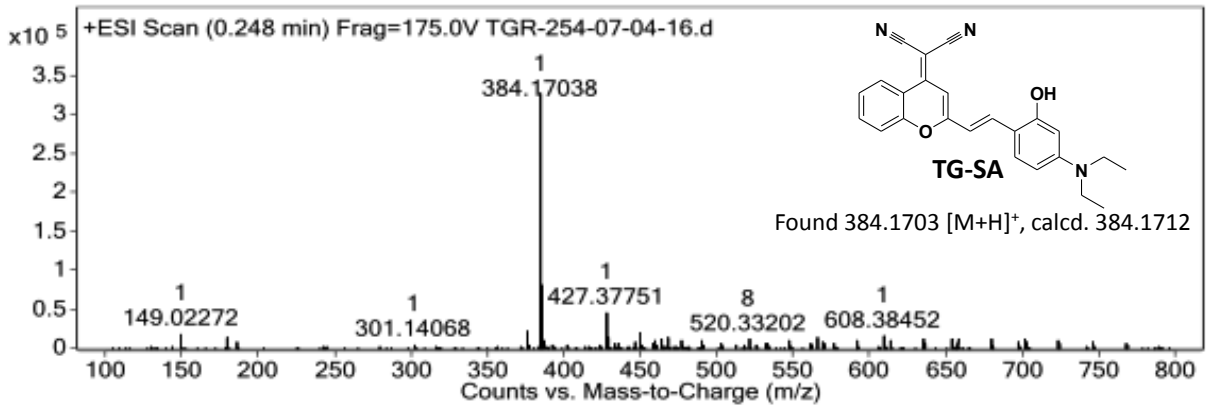


3. HRMS analysis



4. LCMS profile of TG-SA