Electronic Supplementary Information

Cell imaging with red fluorescent light-up probe based on environment-sensitive fluorogen with intramolecular charge transfer characteristics

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Materials: 4,7-Dibromo-2,1,3-benzothiadiazole was purchased from Puyang Huicheng Chemical Co., Ltd (China). Bis(triphenylphosphine)palladium(II) dichloride ($Pd(PPh_3)_2Cl_2$), 2-(tributylstannyl)thiophene, *N*-bromosuccinimide (NBS), copper(I) iodide (CuI), diisopropylamine, ethynyltrimethylsilane, ethynyltrimethylsilane, copper(II) sulphate ($CuSO_4$), sodium ascorbate and albumin of bovine serum were purchased from Sigma-Aldrich. The peptide KRRRQRRKKR (N_3-Ava) was purchased from GenicBio Limited (China). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purcahed from Avanti Polar Lipidd, Inc (USA). All the other chemical reagents and solvents were used as received from commercial sources otherwise specified.

Characterization: NMR spectra were collected on a Bruker Avance 500 NMR spectrometer (500 MHz for ^1H, referenced to TMS at $\delta = 0.00$ ppm). UV-vis spectra were recorded on a Shimadzu UV-1700 spectrometer. Photoluminescence (PL) spectra were measured on a Perkin Elmer LS-55 equiped with a xenon lamp excitation source and a Hamamatsu (Japan) 928 PMT, using 90 degree angle detection for solution samples. All UV-vis and PL spectra were collected at 24 ± 1 ^oC. High resolution mass spectra (HR-MS) were received from VG ZAB-HS system (England). High pressure liquid chromatography (HPLC) was carried out at LUMTECH HPLC (Germany) system using a C_{18} RP column with acetonitrile (0.1% of trifluoroacetic acid (TFA)) and water (0.1% of TFA) as the eluents. Electrospray Ionization Mass Spectrometry (ESI-MS) was performed using MicroTOF Q Electrospray Ionization Mass Spectrometer from Bruker Daltonics. Freeze-drying was carried out using Martin Christ Model Alpha 1-2/LD. Laser light scattering (LLS) measurements were performed using Brookhaven instruments corporation (BIC) 90 plus with $\lambda = 659$ nm, and the particle diameters were calculated by

ZetaPlus Particle Sizing Software Version 3.93. Milli-Q water (18.2 MΩ) was used for all the experiments.

Cell Culture: MCF-7 breast cancer cells were cultured in folate-free Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin streptomycin at 37 °C in a humidified environment containing 5% CO_2. Before experiments, the cells were precultured until confluence was reached.

Cell cytoplasmic lysate: After 80% confluence, the cells (1.5×10^7) were harvested with trypsin-EDTA and then centrifuged at 1500 rpm for 5 min in 50 mL centrifuge tubes. The cells were washed by suspending the cell pellet with 1× PBS. The cells were re-centrifuged at 1500 rpm for 3 min. The supernatant were carefully removed by a pipette. Ice-cold CER I (1500 μL) was added into the tube, and the tube was vortexed vigorously in the highest setting for 15 min to fully suspended the cell pellet. The tube was incubated on ice for further 10 min. Ice-cold CER II (82.5μL) was added to the tube. The tube was then vortexed on the highest setting for 5 seconds, and followed by incubation on ice for 1 min. After additional 5 seconds vortex on the highest setting, the tube was centrifuged at 10000 rpm for 5 min. The supernatant (cytoplasmic extract) was immediately transferred into a clean pre-chilled tube. The cytoplasmic extracts were stored at –80 °C until use.

Titration experiments: Stock DMSO solution of DBT-KRRRQRRKKR was prepared with a concentration of 1×10^{-3} M, which was diluted to be 2 μM with 150 mM PBS solution or cell medium. Aliquots of cell cytoplasmic lysate solution were added to the solutions. The PL spectrum measurements of the resulting solutions were then performed

immediately. The same procedure was also performed upon gradually addition of BSA into DBT-KRRRQRRKKR solution.

Thermal stability of the fluorescence: The thermal stability of the fluorescence was determined from the PL spectra of DBT-KRRRQRRKKR in 150 mM PBS at 37 ^{o}C. After interaction with cell cytoplasmic lysate, the PBS solution of DBT-KRRRQRRKKR was incubated at 37 ^{o}C for 6 days, and the PL spectra were measured daily. For the data evaluation, the fluorescence intensity, taken from the emission maximum, were compared to the values obtained for the fresh solutions (0 day). The thermal stability of the fluorescence was expressed by the ratio of the fluorescence intensity for each sample after incubation at 37 ^{o}C for a designated time interval to its initial value as a function of the incubation time.

pH stability of the fluorescence: The pH stability of the fluorescence was determined from the PL spectra of DBT-KRRRQRRKKR in cell lysate 150 mM PBS at different temperatures.

Cell Imaging: MCF-7 breast cancer cells were cultured in chambers (LAB-TEK, Chambered Coverglass System) at 37 °C. After 80% confluence, the medium was removed and the adherent cells were washed twice with 1× PBS buffer. EDBT or DBT-KRRRQRRKKR in FBS-free DMEM medium at 2 μM was then added to different chambers, respectively. After incubation for 0, 1, 2, 3, and 4 h, the medium was removed. The cells were further incubated with nucleus staining dye Hoechst (5 μg/mL) in FBS-Free medium for 20 min. The cell monolayer was then washed twice with 1× PBS buffer and imaged by confocal laser scanning microscope (CLSM, Zeiss LSM 410, Jena,

Germany) with imaging software (Olympus Fluoview FV1000). The fluorescence signal from the probes was collected above 560 nm upon 488 nm excitation. The nucleus signal was collected between 430 and 470 nm upon 405 nm excitation. The MCF-7 cells were also treated with DBT-KRRRQRRKKR at concentration of 0.5 and 10 μM for 1, 2, 3, and 4 h, respectively. After nucleus staining, the cells were also imaged by CLSM.

Photostability Study. MCF-7 cells incubated with DBT-KRRRQRRKKR, Alexa Fluor 488 or fluorescein were prepared according to the procedures described above. The CLSM images of each sample were recorded at 2 min interval under continuous laser scanning at excitation wavelength of 488 nm with 5 mW laser power. The fluorescence intensity of each image was analyzed by Image Pro Plus software. The photostability of DBT-KRRRQRRKKR, Alexa Fluor 488 or fluorescein was expressed by the ratio of the fluorescence intensity for each sample after excitation for a designated time interval to its initial value as a function of the exposure time.

4,7-Bis(thiophen-2-yl)-2,1,3-benzothiadiazole (1). A solution of 4,7-dibromo-2,1,3-benzothiadiazole (1.76 g, 6.00 mmol), 2-(tributylstannyl)thiophene (5.60 g, 15 mmol) and $Pd(PPh_3)_2Cl_2$ (201.60 mg, 0.30 mmol) in anhydrous THF (30 mL) was stirred at 85 ^oC under argon atmosphere for 15 h. After the mixture was cooled down to room temperature, the precipitate was collected by filtration. The obtained solid was further purified by recrystallization from methanol to yield 4,7-bis(thiophen-2-yl)-2,1,3-benzothiadiazole (1.49 g, yield: 83%) as fiber orange solid. ^1H NMR (500 MHz, $CDCl_3$, ppm) δ: 8.11 (dd, J = 3.7, 0.9 Hz, 2 H), 7.87 (s, 2 H), 7.46 (dd, J = 4.9, 0.9 Hz, 2 H), 7.21 (dd, J = 4.9, 3.7 Hz, 2 H). ^{13}C NMR (125 MHz, $CDCl_3$, ppm) δ: 153.02, 139.75, 128.42, 127.90, 127.21, 126.38, 126.15.

4-(5-Bromothiophen-2-yl)-7-(thiophen-2-yl)-2,1,3-benzothiadiazole (2). 4,7-Bis(thiophen-2-yl)-2,1,3-benzothiadiazole (1.50 g, 5.00 mmol) was dissolved in a mixture of chloroform/acetic acid (50 mL/50 mL). The mixture was kept at 0~10 ^{o}C in an ice bath and shielded from light. NBS (0.71 mg, 4.00 mmol) in 15 mL chloroform was added dropwise with 1 h. The reaction process was monitored by TLC. After 1h, the mixture was poured into aqueous saturated Na_2CO_3 solution. The aqueous layers were extracted with chloromethane. The combined organic extracts were washed with water and dried over $MgSO_4$. After solvent removal, the residue was purified by silica gel column chromatography (hexane/dichloromethane = 8/2) to afford 4-(5-bromothiophen-2-yl)-7-(thiophen-2-yl)-2,1,3-benzothiadiazole (0.64 g, 42%) as a red solid. ^{1}H NMR (500 MHz, $CDCl_3$, ppm) δ: 8.12 (d, *J* = 3.9 Hz, 1 H), 7.86 (d, *J* = 7.8 Hz, 1 H), 7.79 (m, 2 H), 7.46 (d, *J* = 5.1 Hz, 1 H), 7.21 (dd, *J* = 5.1, 3.9 Hz, 1 H), 7.15 (d, *J* = 3.9 Hz, 1 H). ^{13}C NMR (125 MHz, $CDCl_3$, ppm) δ: 152.7, 152.4, 140.9, 139.4, 130.9, 128.3, 127.9, 127.3, 127.2, 126.5, 125.8, 125.3, 125.1, 114.7.

Synthesis of 4-(5-ethynylthiophen-2-yl)-7-(thiophen-2-yl)-2,1,3-benzothiadiazole (3): To a solution of 4-(5-bromothiophen-2-yl)-7-(thiophen-2-yl)-2,1,3-benzothiadiazole (189.5 mg, 0.5 mmol), copper iodide (9.5 mg, 0.05 mmol) and $Pd(PPh_3)_2Cl_2$ (35.1 mg, 0.05 mmol) in diisopropylamine (30 mL) under argon atmosphere was added ethynyltrimethylsilane (210 μL, 1.5 mmol) via syringe. The reaction was performed at 70 ^{o}C overnight before the solvent was removed under reduced pressure. The residue was redissolved in dichloromethane (100 mL) and run through a short silica gel column to remove catalysts. The crude product was mixed with potassium hydroxide (0.56 g, 10 mmol), THF (20 mL), methanol (10 mL) and water (5 mL) in a round bottle flask. The

mixture was stirred at room temperature under argon atmosphere for 1 h. After solvent removal, the residue was subsequently redissolved in dichloromethane, washed with water and dried over $MgSO_4$. The crude product was purified by silica gel column chromatography using hexane/dichloromethane (8/2) as eluent to 4-(5-ethynylthiophen-2-yl)-7-(thiophen-2-yl)-2,1,3-benzothiadiazole as red solid (140.9 mg, yield: 81%). ^{1}H NMR (500 MHz, $CDCl_3$, ppm), δ: 8.11 (m, 1 H), 7.94 (m, 1 H), 7.84 (m, 2 H), 7.46 (m, 1 H), 7.34 (m, 1 H), 7.20 (m, 1 H), 3.48 (s, 1 H). ^{13}C NMR (125 MHz, $CDCl_3$, ppm) δ: 152.46, 140.88, 139.17, 133.91, 128.11, 127.80, 127.52, 127.16, 126.88, 126.63, 125.96, 125.59, 125.00, 123.12, 82.94, 77.11.

Synthesis of DBT-KRRRQRRKKR. To a solution of 4-(5-ethynylthiophen-2-yl)-7-(thiophen-2-yl)-2,1,3-benzothiadiazole (1.6 mg, 5.0 μmol) and alkyne-modified KRRRQRRKKR (11.1 mg, 7.0 μmol) in DMSO (1 mL) was added 0.1 M $CuSO_4$ (50 μL) and 1 M sodium ascorbate (100 μL) aqueous solution. The mixture was stirred at room temperature for 2 days. After filtration through 0.45 μm filter, the filtrate was further purified by HPLC using acetonitrile (0.1% of TFA) and water (0.1% of TFA) as the eluents. After freeze-drying, DBT-KRRRQRRKKR was obtained as red fibrous solid (3.6 mg, yield: 38%). ^{1}H NMR (500 MHz, DMSO-d_6, ppm), δ: 8.60 (br, 3 H), 8.53 (br, 2 H), 8.17–8.05 (br, 9 H), 7.85–7.54 (m, 10 H), 7.35–6.91 (m, 16 H), 6.52 (br, 10 H), 4.40–.09 (br, 8 H), 3.81 (br, 1 H), 3.34 (br, 31 H), 3.07 (br, 8 H), 2.99 (br, 2 H), 2.73 (br, 4 H), 2.57 (br, 1 H), 2.36 (br, 1 H), 2.11 (br, 4 H), 1.84 (br, 2 H), 1.66 (br, 6 H), 1.50 (br, 10 H), 1.36–1.26 (br, 4 H). EIMS: M/Z = 639.9921 $[M]^{3+}$.

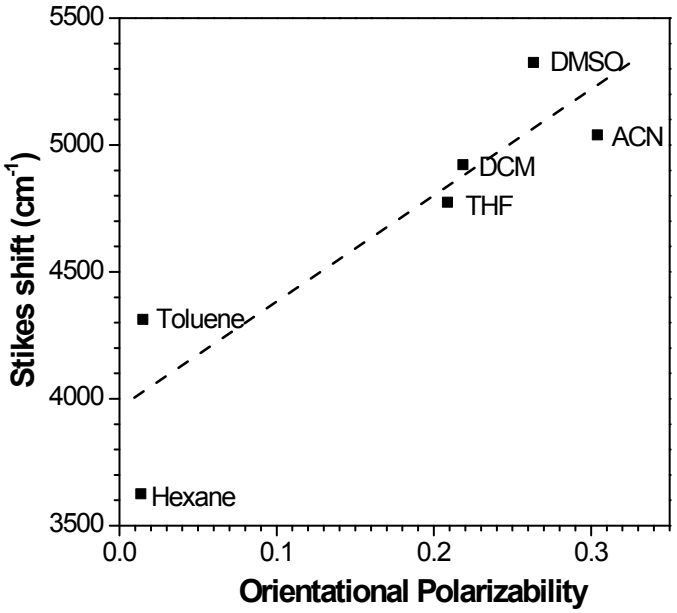


Fig. S1 Lipper plots of EDBT in different solvent. Orientational polarizability (f) is calculated based on refractive index (n) and dielectric constant (ε),

where $f = |\frac{n^2-1}{2n^2+1} - \frac{\varepsilon-1}{2\varepsilon+1}|$.

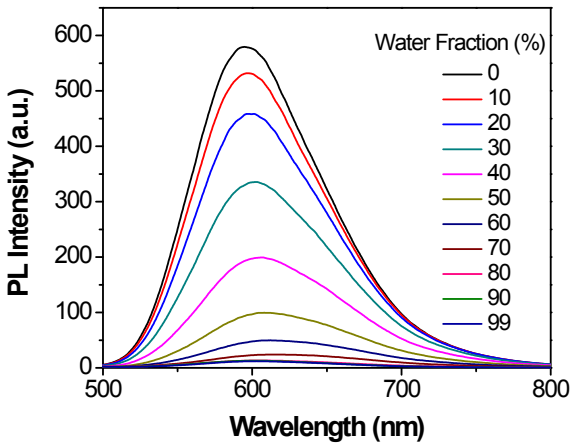


Fig. S2 PL spectra of DBT in DMSO-water mixture with different water fraction (f_w).

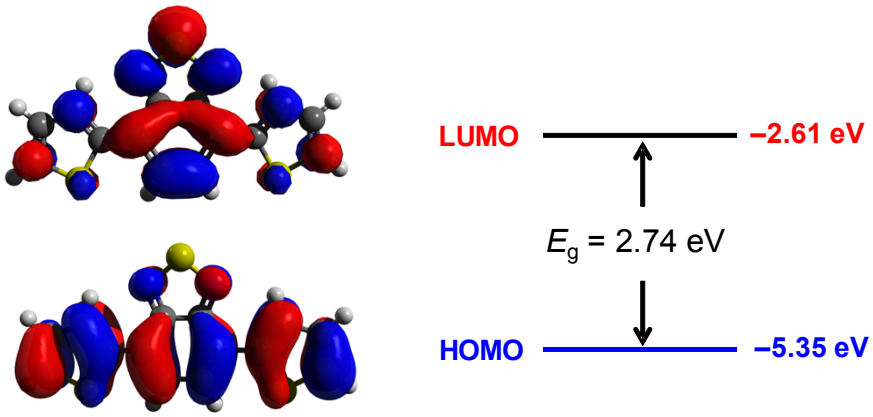
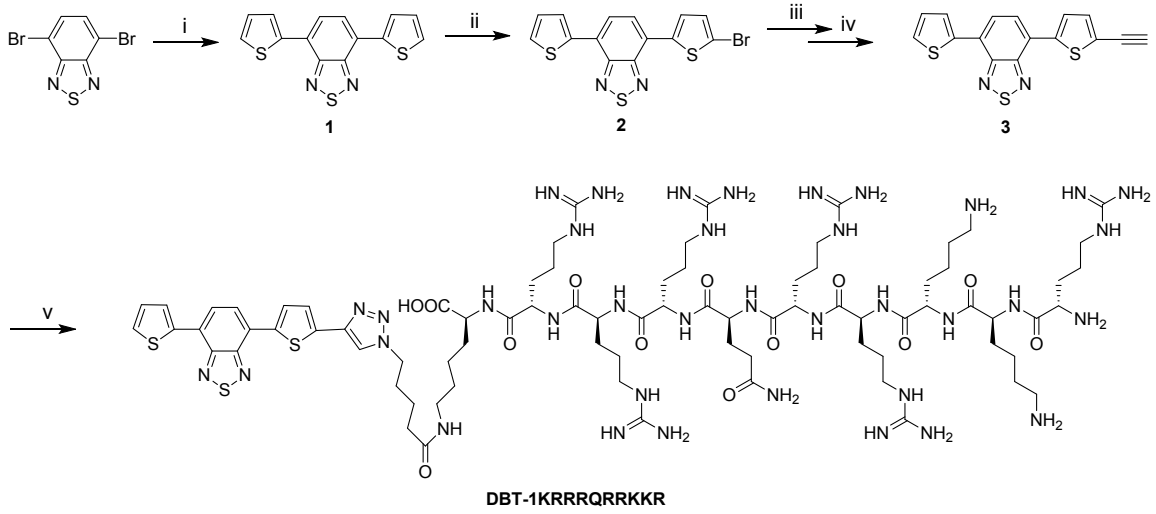


Fig. S3 The frontier molecular HOMO and LUMO orbitals of DBT.



Scheme S1 Synthetic route to DBT-KRRRQRRKKR. *Reagents and conditions*: i) $Pd(PPh_3)_2Cl_2$, 2-(tributylstannyl)thiophene , THF, 85 °C, 15 h; ii) NBS, chloroform/acetic acid, room temperature, 2 h; iii) CuI, $Pd(PPh_3)_2Cl_2$, trimethylsilylaceylene, $^{i}Pr_2NH$/THF, 70 °C, overnight; iv) KOH, THF/MeOH/H_2O, room temperature, 1 h; v) $CuSO_4$, sodium ascorbate, DMSO/water, room temperature, 2 days.

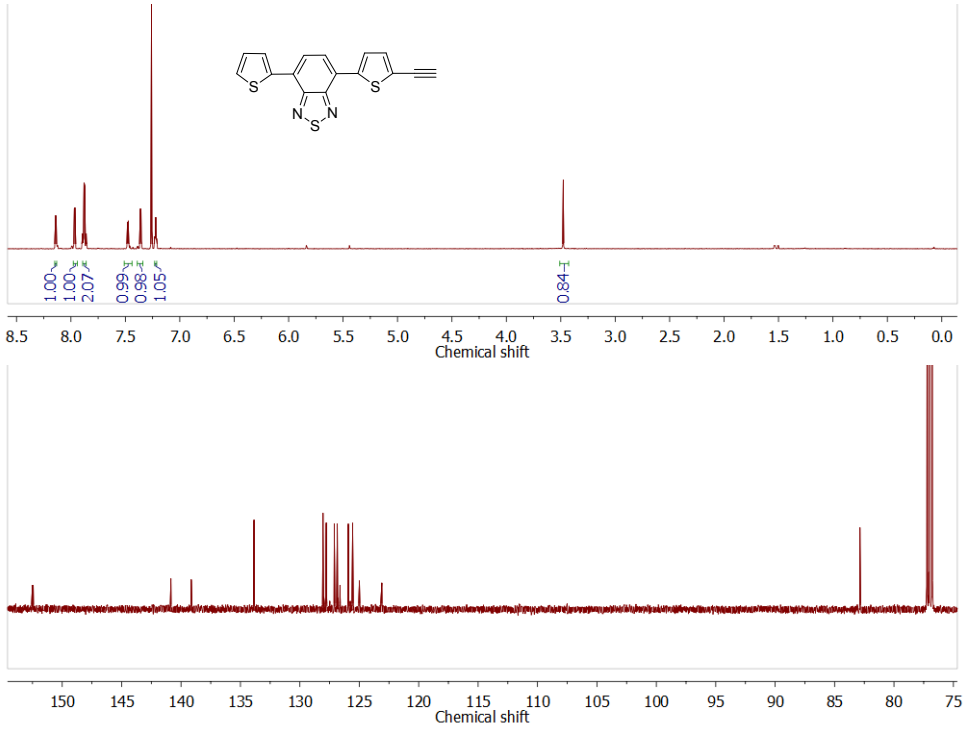


Fig. S4 ^{1}H NMR (top) and ^{13}C NMR (bottom) spectra of compound **3** in $CDCl_3$.

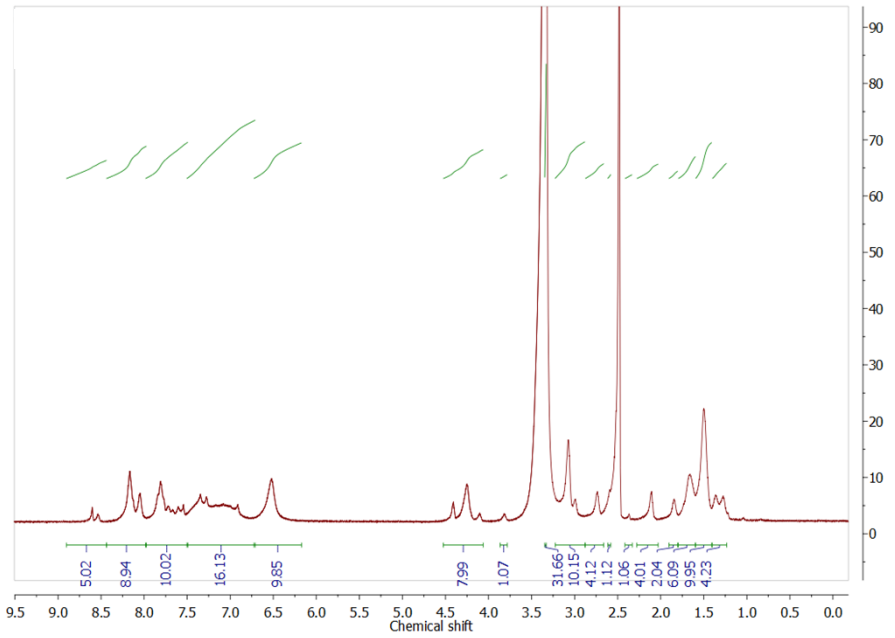


Fig. S5 ^{1}H NMR spectrum of DBT-KRRRQRRKKR in DMSO-d_6.

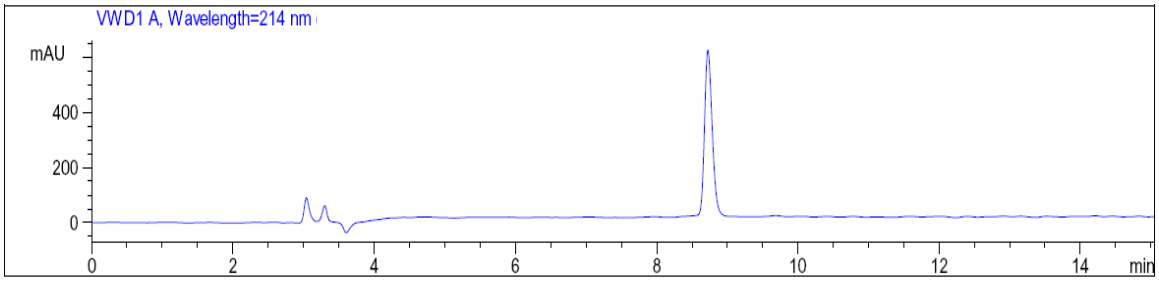


Fig. S6 HPLC trace of DBT-KRRRQRRKKR (A_{254}).

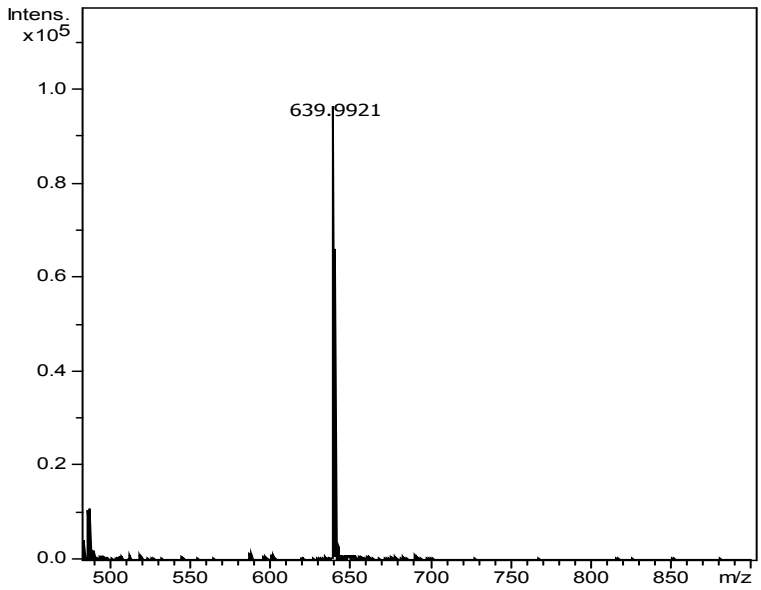


Fig. S7 EIMS profile of DBT-KRRRQRRKKR.

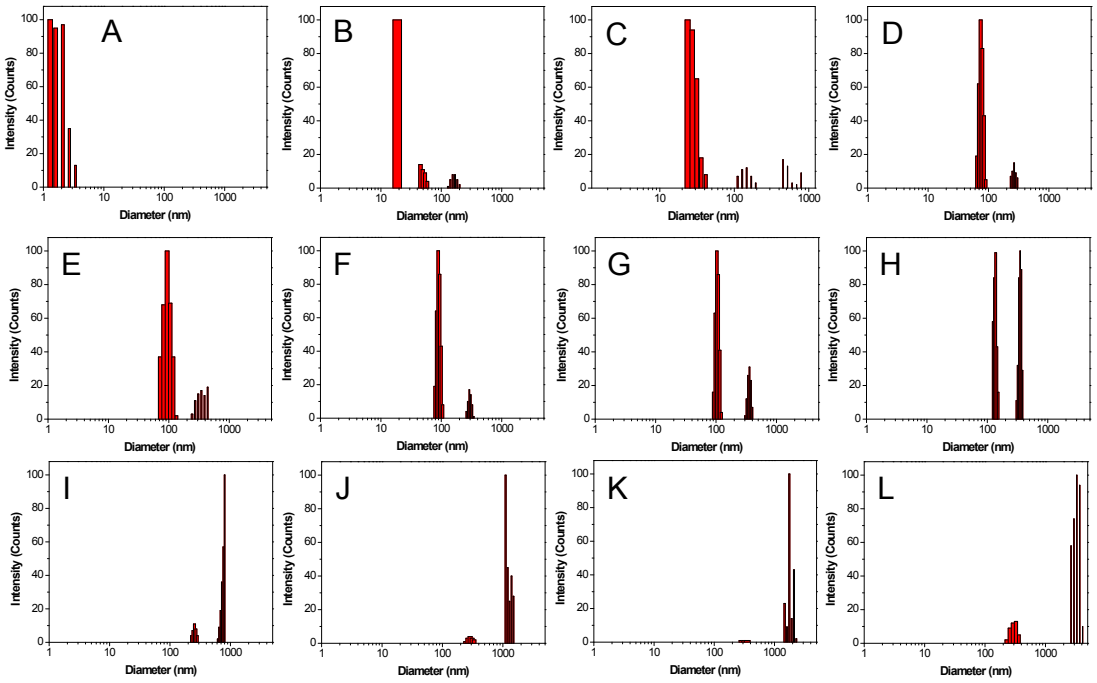


Fig. S8 hydrodynamic diameters of DBT-KRRRQRRKKR in 150 mmol PBS solution with increasing addition of cell cytoplasmic lysate from (A) to (L).

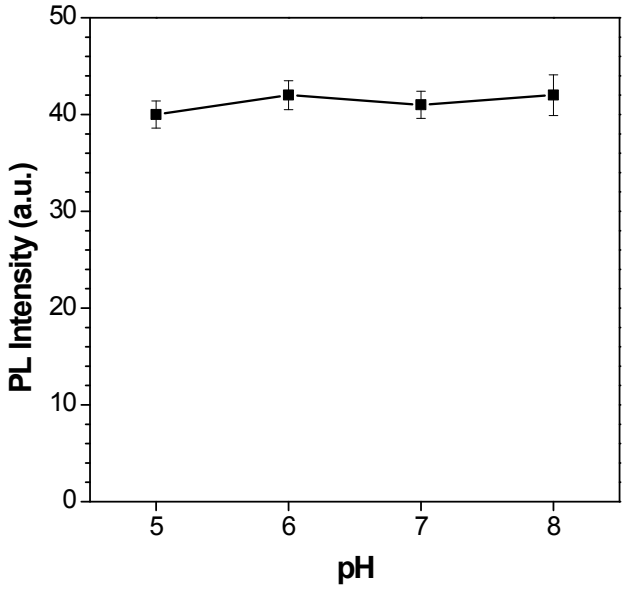


Fig. S9 PL spectra of DBT-KRRRQRRKKR in cell lysate upon changing pH value.

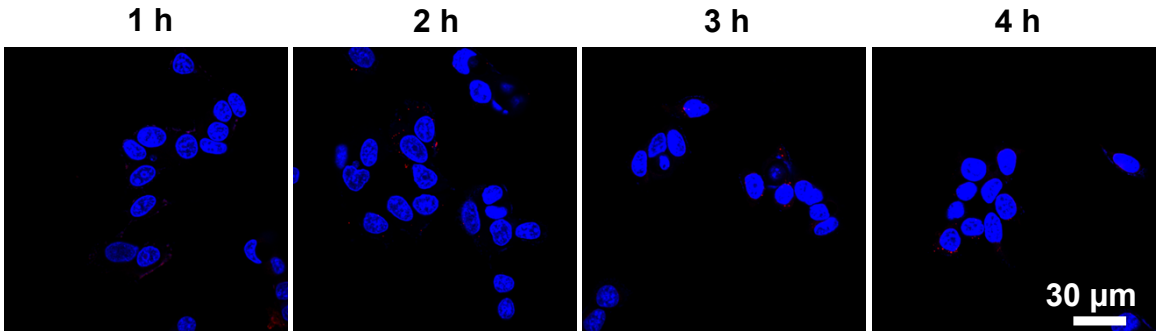


Fig. S10 CLSM images of MCF-7 cells treated with EDBT concentration of 2 μM for 1, 2, 3, and 4 h, respectively. The nucleus is labelled by Hoechest. All the images share the same scale bar.

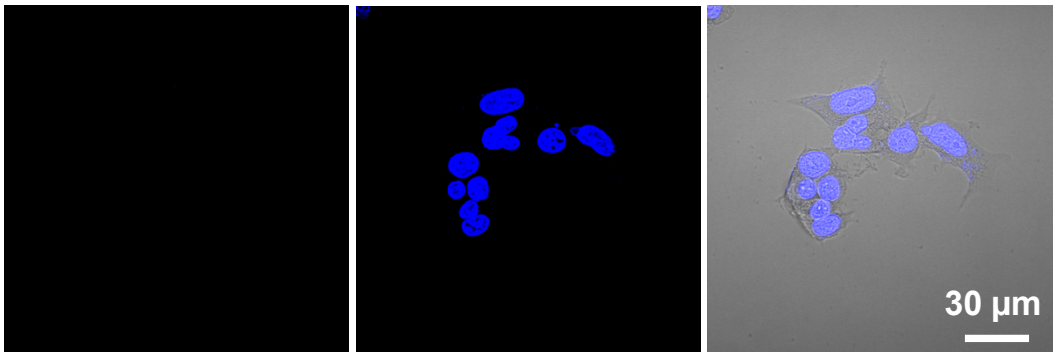


Fig. S11 CLSM images of MCF-7 cells immediately taken after addition DBT-KRRRQRRKKR concentration of 2 μM (0 h). The nucleus is pre-labelled by Hoechest for 20 min. All the images share the same scale bar.

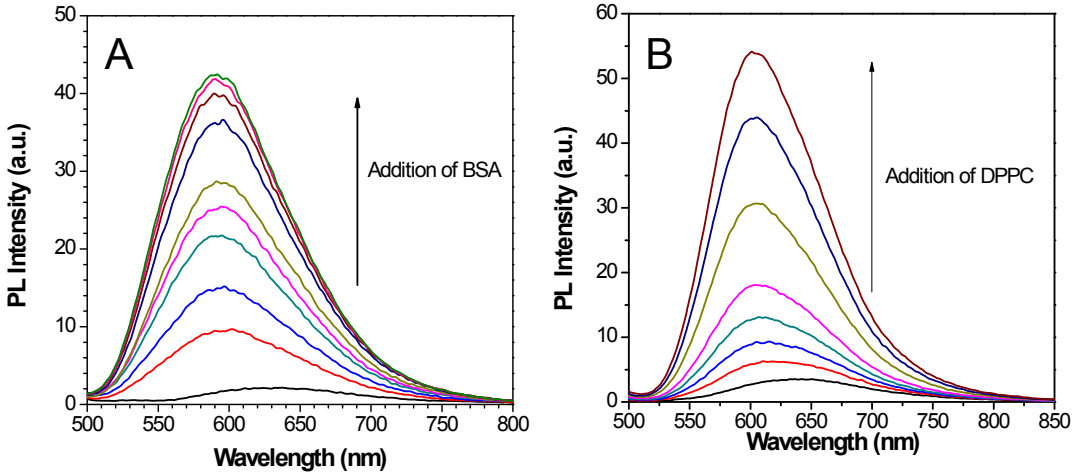


Fig. S12 PL spectra of DBT-KRRRQRRKKR in 1 × PBS solution upon addition of BSA (A) or DPPC (B).

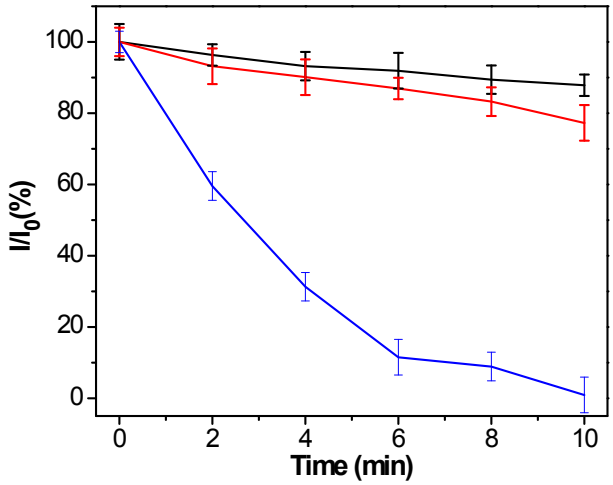


Fig. S13 Photostability comparison among DBT-KRRRQRRKKR (black), Alexa Fluor 488 (red) and fluorescein (blue) in MCF-7 cancer cells upon continuous laser excitation at 488 nm with a laser power of 5 mW. I_0 is the initial fluorescence intensity and I is the fluorescence intensity of each sample at various time points after continuous scanning.

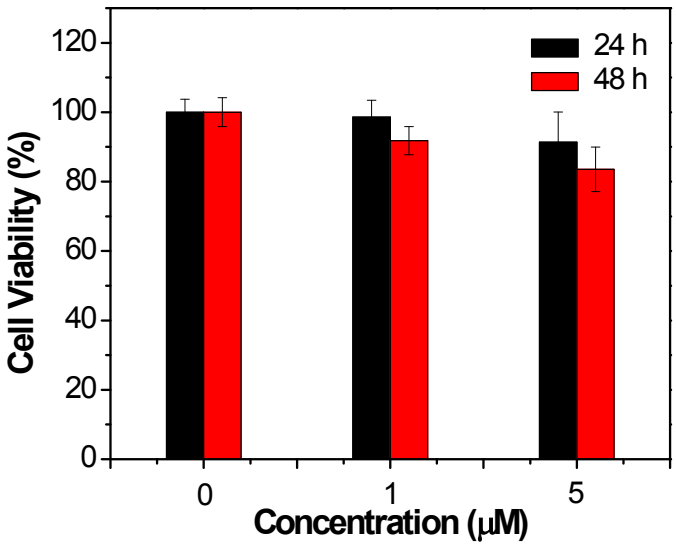


Fig. S14 Cell viability of MCF-7 cells after incubation with DBT-KRRRQRRKKR at different concentraitons for 24, and 48 h, respectively.