Electronic Supplementary Material (ESI) for Polymer Chemistry

Supporting information

A novel method of preparing AIE dye based cross-linked fluorescent polymeric nanoparticles for cell imaging

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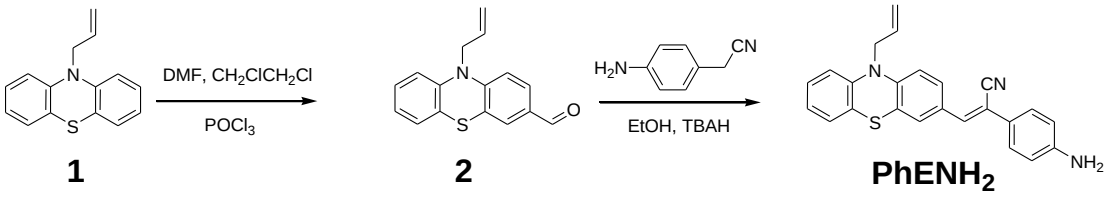
1. Experimental

1.1 Materials and measurements

Phenothiazine, 3-bromoprop-1-ene, N,N-dimethylformamide (DMF), 1,2-dichloroethane, phosphoryl chloride, 4-aminobenzyl cyanide, tetrabutylammonium hydroxide (0.8M in methanol), N,N-dimethylacetamide (DMAc), 4,4'-Oxydiphthalic anhydride purchased from Alfa Aesar were used as received. Poly(ethylene glycol) monomethyl ether methacylate (PEGMA, Mn = 950 Da) was purchased from ALDRICH company. All other agents and solvents were purchased from commercial sources and used directly without further purification. Tetrahydrofuran (THF) was distilled from sodium/benzophenone. Ultra-pure water was used in the experiments.

^{1}H NMR spectra were measured on a JEOL 400 MHz spectrometer [$CDCl_3$ or d_6-DMSO as solvent and tetramethylsilane (TMS) as the internal standard]. HRMS was obtained on Shimadzu LCMS-IT-TOF high resolution mass spectrometry. UV-Visible absorption spectra were recorded on UV/Vis/NIR Perkin-Elmer lambda750 spectrometer (Waltham, MA, USA) using quartz cuvettes of 1 cm path length. Fluorescence spectra were measured on a PE LS-55 spectrometer with a slit width of 3 nm for both excitation and emission. The FT-IR spectra were obtained in a transmission mode on a Perkin-Elmer Spectrum 100 spectrometer (Waltham, MA, USA). Typically, 8 scans at a resolution of 1 cm^{-1} were accumulated to obtain one spectrum. Transmission electron microscopy (TEM) images were recorded on a JEM-1200EX microscope operated at 100 kV, the TEM specimens were made by placing a drop of the nanoparticles suspension on a carbon-coated copper grid. The size distribution and zeta potential measurement of **RAFT-RO-20(40)** FPNs in phosphate buffer solution (PBS) were determined using a zeta Plus apparatus (ZetaPlus, Brookhaven Instruments, Holtsville, NY). Gel permeation chromatography (GPC) analyses of polymers were performed using DMF as the eluent. The GPC system was a Shimadzu LC-20AD pump system comprising of an auto injector, a MZ-Gel SDplus 10.0 mm guard column (50×8.0 mm, 10^2 Å) followed by a MZ-Gel SDplus 5.0 μm bead-size columns (50-10^6 Å, linear) and a Shimadzu RID-10A refractive index detector. The system was calibrated with narrow molecular weight distribution polystyrene standards ranging from 200 to 10^6 g mol^{-1}.

1.2 Synthesis and characterization of $PhENH_2$



Scheme S1 Synthetic route of **$PhENH_2$**.

The intermediate 10-allyl-10H-phenothiazine (**1**) was synthesized according to the literature.[1]

In a 500 mL flask, $POCl_3$ (24.5 g, 0.16 mol) and DMF (7.8 g, 0.106 mol) were added to CH_2ClCH_2Cl (20 mL) at 0 °C. After stirring the reaction mixture for 30 minutes, the intermediate **1** (5.0 g, 21 mmol) was added. The reaction mixture was heated at 90 °C overnight. The crude product was added dropwise into 600 mL cool water and extracted with ethyl acetate. The pure 10-allyl-10H-phenothiazine-3-carbaldehyde (**2**) was isolated by flash column chromatography using a 1:1 mixture of dichloromethane and petroleum ether as an eluent (3.3 g, yield 59%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.49 (quint, 2H, J = 2.0 Hz), 5.19-5.41 (m, 2H), 5.93-6.06 (m, 1H), 6.81-6.88 (m, 2H), 6.89-6.97 (m, 1H), 7.01-7.12 (m, 2H), 7.49-7.57 (m, 2H), 9.75 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 190.07, 149.80, 142.85, 131.89, 131.16, 130.42, 127.00, 122.18, 118.14, 115.94, 114.80, 51.91; IR (cm^{-1}): 3080, 3061, 3016, 2933, 2829, 2742, 1676, 1597, 1572, 1549, 1468, 1440, 1364, 1314, 1290, 1260, 1215, 1203, 1156, 1114, 922, 898, 804, 744; HRMS calcd. for $C_{16}H_{13}NOS$, $[M+H]^+$: 268.0791, found 268.0787.

A solution of **2** (0.534 g, 2.0 mmol) and 2-(4-aminophenyl)acetonitrile (0.29 g, 2.2 mmol) in ethanol (20 mL) was stirred at room temperature. Then terabutyl ammonium hydroxide solution (0.8 M, 5 drops) was added and the mixture was heated to reflux for 2 h precipitating a red solid. The reaction mixture was cooled to room temperature and filtered, washed with ethanol for several times obtaining a dark red solid **$PhENH_2$** (0.41 g, yield 54%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.86 (s, 2H), 4.46 (s, 2H), 5.18-5.39 (m, 2H), 5.90-6.05 (m, 1H), 6.68 (d, 2H, J = 8.4 Hz), 6.76-6.94 (m, 3H), 7.01-7.11 (m, 2H), 7.15 (s, 1H), 7.38-7.47 (m, 3H), 7.65 (dd, 1H, J_1 = 8.4 Hz, J_2 = 2.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 147.33, 145.63, 143.51, 137.19, 132.34, 127.51, 126.96, 123.10, 122.26, 117.94, 115.22, 115.20, 108.88, 51.55; IR (cm^{-1}): 3340, 3033, 2920, 2846, 2206, 1622, 1605, 1576, 1516, 1476, 1404, 1365, 1285, 1256, 1182, 1149, 826; HRMS calcd. for $C_{24}H_{19}N_3S$, $[M+Na]^+$: 382.1372, found 382.1373.

1.3 Preparation of RAFT-RO-20(40) FPNs

In a typical synthesis of **RAFT-RO-20**, PEGMA (0.63 g, 0.66 mmol), **$PhENH_2$** (0.028 g,

0.074 mmol), CTA (9.7 mg, 0.037 mmol), AIBN (3.0 mg) and dioxane (3 mL) were introduced in schlenk tube and purged by nitrogen flow for 30 min. The above mixture was put into an oil bath maintained at 70 °C for 12h. Then stop the reaction of polymerization, and the solution was allowed to cool to room temperature. Followed by adding 4,4'-oxydiphthalic anhydride (11.5 mg, 0.037 mmol) which was dissolved in 3 mL DMAc. The above mixture was stirred under air atmosphere at room temperature for 2h. Then the reaction of polymerization was stopped and the purified polymer was obtained via precipitation from the above mixed solvent to petroleum ether for three times, and then dried under vacuum for further characterization. IR (cm^{-1}): 2866, 1729, 1455, 1351, 1296, 1249, 1095, 1038, 947, 849. The synthetic procedure of **RAFT-RO-40** was similar to that of **RAFT-RO-20**, whereas the amount of CTA was changed to 4.85 mg. IR (cm^{-1}): 2866, 1730, 1454, 1348, 1295, 1248, 1098, 1038, 945, 847.

1.4 Cytotoxicity of RAFT-RO-20(40) FPNs

Cell morphology was observed to examine the effects of **RAFT-RO-20(40)** FPNs to A549 cells. Briefly, cells were seeded in 6-well microplates at a density of 1×10^5 cells mL^{-1} in 2 mL of respective media containing 10% fetal bovine serum (FBS). After cell attachment, plates were washed with PBS and cells were treated with complete cell culture medium, or different concentrations of **RAFT-RO-20(40)** FPNs prepared in 10% FBS containing media for 24 h. Then all samples were washed with PBS three times to remove the uninternalized nanoparticles. The morphology of cells was observed by using an optical microscopy (Leica, Germany), the overall magnification was ×100.

The cell viability of **RAFT-RO-20(40)** FPNs on A549 cells was evaluated by cell counting kit-8 (CCK-8) assay. Briefly, cells were seeded in 96-well microplates at a density of 5×10^4 cells mL^{-1} in 160 μL of respective media containing 10% FBS. After 24 h of cell attachment, the cells were incubated with 10, 20, 40, 80, 120 μg mL^{-1} **RAFT-RO-20(40)** FPNs for 8 and 24 h. Then nanoparticles were removed and cells were washed with PBS three times. 10 μL of CCK-8 dye and 100 μL of DMEM cell culture medium were added to each well and incubated for 2 h at 37 °C. Plates were then analyzed with a microplate reader (VictorIII, Perkin-Elmer). Measurements of formazan dye absorbance were carried out at 450 nm, with the reference wavelength at 620 nm. The values were proportional to the number of live cells. The percent reduction of CCK-8 dye was compared to controls (cells not exposure to **RAFT-RO-20(40)** FPNs), which represented 100%

CCK-8 reduction. Three replicate wells were used per microplate, and the experiment was repeated three times. Cell survival was expressed as absorbance relative to that of untreated controls. Results are presented as mean ± standard deviation (SD).

1.5 Confocal microscopic imaging of cells using RAFT-RO-20(40) FPNs

A549 cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} of streptomycin. Cell culture was maintained at 37 °C in a humidified condition of 95% air and 5% CO_2 in culture medium. Culture medium was changed every three days for maintaining the exponential growth of the cells. On the day prior to treatment, cells were seeded in a glass bottom dish with a density of 1×10^5 cells per dish. On the day of treatment, the cells were incubated with **RAFT-RO-20(40)** FPNs at a final concentration of 10 μg mL^{-1} for 3 h at 37 °C. Afterward, the cells were washed three times with PBS to remove the **RAFT-RO-20(40)** FPNs and then fixed with 4% paraformaldehyde for 10 min at room temperature. Cell images were taken with a confocal laser scanning microscope (CLSM) Zesis 710 3-channel (Zesis, Germany) with the excitation wavelength of 405 nm.

2. Results

Fig. S1 The optimized conformation structure (A) and calculated spatial electron distributions of HOMO (B) and LUMO (C) of **$PhENH_2$**.

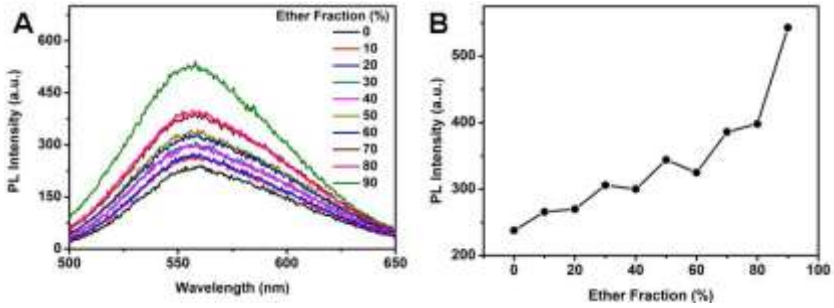


Fig. S2 The AIE characteristic of **$PhENH_2$** (λ_{Ex} = 490 nm): (A) PL spectra of **$PhENH_2$** in DMF–ether mixtures with different ether fractions; (B) the changes in PL peak intensity of the compound in different ether fraction mixtures.

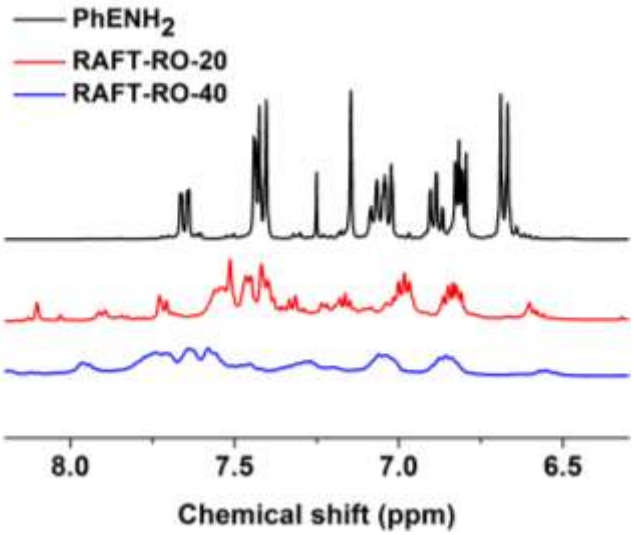


Fig. S3 ^{1}H NMR spectra of **PhENH$_2$**, **RAFT-RO-20** and **RAFT-RO-40** dissolved in d_6-DMSO.

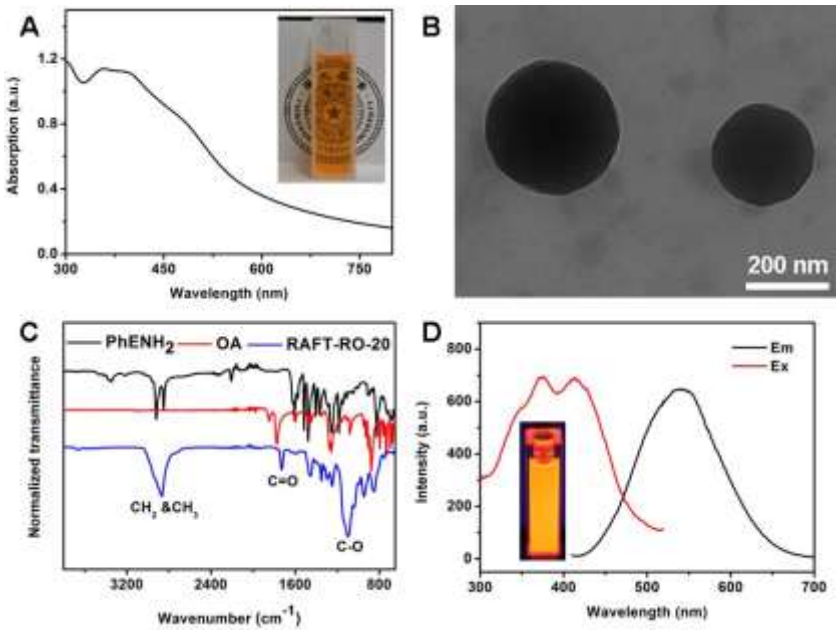


Fig. S4 Characterization of **RAFT-RO-20** FPNs: (A) normalized IR spectra of **PhENH$_2$**, **OA** and **RAFT-RO-20** copolymers, strong stretching vibration bands of C=O which located at 1729 cm^{-1} and C-O stretching vibration bands which located at 1095 cm^{-1} were observed in the sample of **RAFT-RO-20** FPNs, suggesting **RAFT-RO-20** FPNs were formed; (B) UV-Vis spectrum of **RAFT- RO-20** FPNs, inset is the visible image of **RAFT-RO-20** FPNs in water using the logo of "Tsinghua University" as background; (C) TEM image of **RAFT-RO-20** FPNs dispersed in water, scale bar = 200 nm; (D) fluorescence excitation and emission spectra of **RAFT-RO-20** FPNs, inset is the fluorescent image of **RAFT-RO-20** FPNs taken at 365 nm UV light.

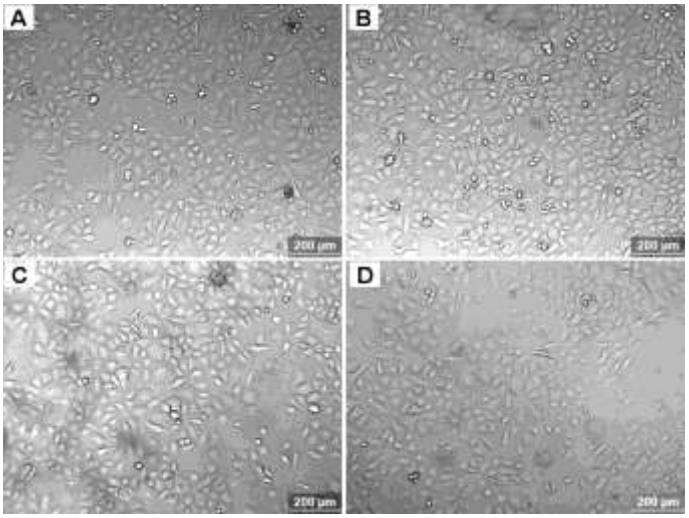


Fig. S5 Optical microscopy images of A549 cells incubated with different concentrations of **RAFT-RO-20** FPNs for 24 h: (A) control cells, (B) 10 μg mL^{-1}, (C) 80 μg mL^{-1}, (C) 120 μg mL^{-1}.

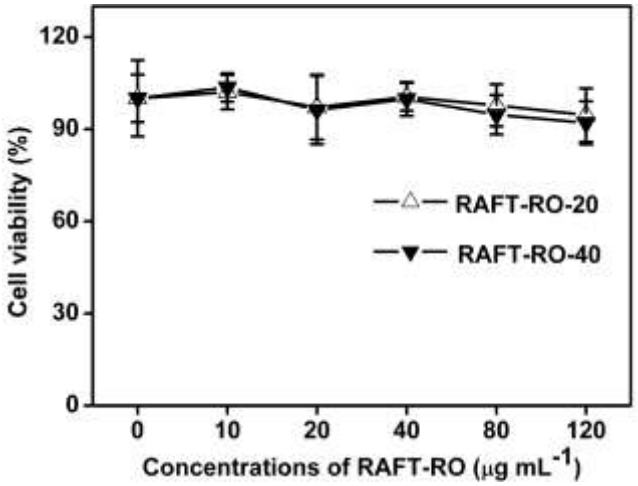


Fig. S6 cell viability of **RAFT-RO-20(40)** FPNs with A549 cells for 12 h.

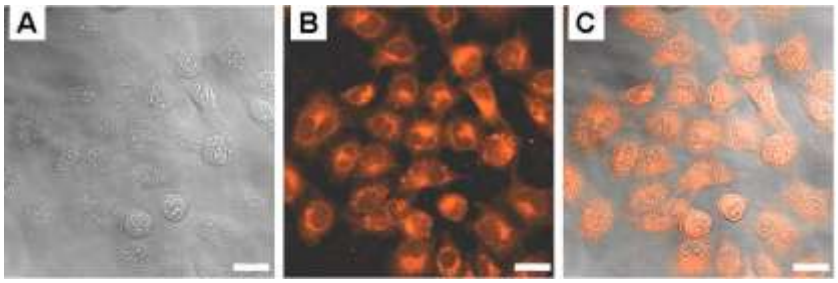


Fig. S7 CLSM images of A549 cells incubated with 10 μg mL^{-1} of **RAFT-RO-20** FPNs for 3 h. (A) bright field, (B) excited with 405 nm laser, (C) merge image of A and B. Scale bar = 20 μm.

Reference

1. K.-H. Gan, C.-J. Jhong, Y.-J. Shue and S.-C. Yang, *Tetrahedron*, 2008, **64**, 9625-9629.