Supporting information

Renewable itaconic acid based cross-linked fluorescent polymeric nanoparticles for cell imaging

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1. Experimental

1.1 Materials and measurements

Phenothiazine, 1-bromooctadecane, N,N-dimethylformamide (DMF), 1,2-dichloroethane, phosphoryl chloride, 2-(4-bromophenyl)acetonitrile, 4-vinylphenylboronic acid, Aliquat 336, tetrakis(triphenylphosphine) palladium(0), tetrabutylammonium hydroxide (0.8M in methanol), N,N-dimethylacetamide (DMAc), azobisisobutyronitrile purchased from Alfa Aesar were used as received. All other agents and solvents were purchased from commercial sources and used directly without further purification. Ultra-pure water was used in the experiments.

$^1$H NMR and $^{13}$C 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]. Standard MS was obtained on ZAB-HS 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 fluorescence quantum yield values ($\Phi_F$) of the FPNs were estimated using quinine sulfate in 0.1 N H$_2$SO$_4$ ($\Phi_F = 54.6\%$) as standard. Fluorescence lifetime data were measured on FLS 920 lifetime and steady state spectrometer. 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 the 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 PhE2
Intermediates $2^1$ and $3^2$ were synthesized according to our previous literatures. For the synthesis of PhE2, a solution of $2$ (0.28 g, 0.58 mmol) and $3$ (0.31 g, 1.42 mmol) in ethanol (10 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 hours precipitating an orange solid. The reaction mixture was cooled to room temperature and filtered, washed with ethanol for several times obtaining an orange solid PhE2 (0.45 g, yield 90%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm): 0.87 (m, 3H), 1.20-1.33 (m, 28H), 1.45 (quint, 2H), 1.82 (quint, 2H), 3.86 (t, 2H), 5.29 (d, 2H), 5.91 (d, 2H), 6.75 (q, 2H), 6.87 (t, 2H), 7.38 (s, 2H), 7.45-7.53 (m, 6H), 7.58 (d, 4H), 7.62-7.73 (m, 8H), 7.83 (dd, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ (ppm): 145.7, 141.2, 140.1, 136.3, 133.6, 128.7, 127.2, 126.3, 123.8, 118.4, 115.4, 114.4, 108.8, 100.0, 48.2, 32.0, 29.5, 26.9, 26.7, 22.8, 14.2; MS (FAB) calcd. for C$_{64}$H$_{67}$N$_3$S 910, found 911 [M+H]$^+$.  

1.3 Preparation of PhE2-IA and PhE-IA FPNs

For synthesis of PhE2-IA FPNs (see scheme 2), PhE2 (14 mg, 0.015 mmol), IA (39 mg, 0.30 mmol), AIBN (5.0 mg), and dioxane (6 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 80 °C for 12h. Afterwards, the cross-linking polymerization was stopped, and dialyzed against tap water for 24 h and ethanol for 6 h using 7000 Da $M_w$ cutoff dialysis membranes. Finally, the solution in dialysis bag was carried out by freeze-drying to obtain PhE2-IA FPNs. The synthesis of PhE-IA FPNs (see scheme S2) was similar to that of PhE2-IA FPNs, while using PhE (PhE was synthesized according to our previous reference 2) as the AIE monomer instead of PhE2, and the amounts of PhE was adjusted to 10 mg (0.015 mmol), other conditions remain unchanged.
1.4 Cytotoxicity of PhE2-IA and PhE-IA FPNs

Cell morphology was observed to examine the effects of PhE2-IA and PhE-IA FPNs to A549 cells. Briefly, cells were seeded in 6-well microplates at a density of $1 \times 10^5$ 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 PhE2-IA and PhE-IA 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 $\times 100$.

The cell viability of PhE2-IA and PhE-IA 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 $5 \times 10^4$ 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$^{-1}$ PhE2-IA and PhE-IA 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 the 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 PhE2-IA 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%
CO₂ 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 1×10⁵ cells per dish. On the day of treatment, the cells were incubated with PhE2-IA FPNs at a final concentration of 20 µg mL⁻¹ for 3 h at 37 °C. Afterward, the cells were washed three times with PBS to remove the PhE2-IA 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 488 nm.

2. Results

![Fig. S1](image)

**Fig. S1** The calculated lowest energy spatial conformation of PhE2.

![Fig. S2](image)

**Fig. S2** The AIE characteristic of PhE2 (λₑₓ = 560 nm): PL spectra of PhE2 in DMF–water mixtures with different water fractions; the inset is the change in PL peak intensity of the
compound in different water fraction mixtures.

**Fig. S3** $^1$H NMR spectrum of PhE-IA dissolved in $d_6$-DMSO.

**Fig. S4** (A) UV-Vis spectrum of PhE-IA FPNs, inset is the visible image of PhE-IA FPNs in water with the logo of “Tsinghua University” as background; (B) Fluorescence excitation (Ex) and emission (Em) spectra of PhE-IA FPNs, inset is the fluorescent image of PhE-IA FPNs taken at 365 nm UV light.

**Fig. S5** Time-resolved emission-decay curves of (A) PhE2-IA and (B) PhE-IA at different concentrations.
Table S1 Fluorescence lifetime data of PhE2-IA and PhE-IA at different concentrations

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<th>Log[C] (mg mL⁻¹)</th>
<th>τ₁ (ns)ᵃ</th>
<th>A₁ᵇ</th>
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<th>A₂ᵇ</th>
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ᵃ Fluorescence lifetime.ᵇ Fractional contribution.ᶜ Weighted mean lifetime

Fig. S6 Biocompatibility evaluations of PhE2-IA FPNs. (A-C) optical microscopy images of A549 cells incubated with different concentrations of PhE2-IA FPNs for 24 h, (A) control cells, (B) 20 μg mL⁻¹, (C) 80 μg mL⁻¹, (D) cell viability of PhE2-IA FPNs with A549 cells. The biocompatibility evaluation suggested that PhE2-IA FPNs were biocompatible enough for
biomedical applications.

**Fig. S7** Cell viability of PhE-IA FPNs with A549 cells. The biocompatibility evaluation suggested that PhE-IA FPNs were biocompatible enough for biomedical applications.

**Reference**
