Electronic Supporting Information

A Heptamethine Cyanine with Meso-*N*-Induced Rearrangement for Acid-Activated Tumor Imaging and Photothermal Therapy

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1. Reagents and materials

4-Bromophenylhydrazine hydrochloride (97%), 3-methyl-2-butanone (98%), Cu $(NO_3)_2 \cdot 2.5H_2O$ (99%), iodomethylbenzene (99%), Triphenylamine (98%), p-anisidine (98%), 2-chloro-3-(hydroxymethylidene), cyclohexene-1-carbaldehyde aniline (99%) and hydrazine monohydrate were obtained from HEOWNS and used without further purification. Analytical thin layer chromatography (TLC) was performed on Yantai chemical industry silica gel plates. Dulbecco's modified Eagle's medium (DMEM) with 10% FBS (fetal bovine serum), phosphate-buffered solution (PBS), trypsin and cell counting kit-8 (CCK-8) were obtained from Beijing Solarbio Science & Technology Co. Ltd. The other solvents were purchased from commercial suppliers and were used as received.

2. Instruments

^{1}H Nuclear magnetic resonance (NMR) spectra were recorded on BRUKER DMX 500 spectrometer. DMSO-d6 was used as solvent and tetramethylsilane (TMS) was used as internal reference respectively. Mass spectra were recorded with XEVO-G2QTOF (Waters, USA). Fluorescence spectra were measured with fluorescence spectrophotometer FluoroMax-4 NIR (Horiba Jobin Yvon, USA). UV-visible spectra were measured with UV-2600 (Shimadzu, Japan). The pH values were measured by using a pH meter (Mettler Toledo S40K). The cell images were taken by using a fluorescence microscope (EVOS f1, AMG). Transmission electron microscopy (TEM) micrographs were recorded on a HT-7700 (Hitachi, Japan). The Dynamic light scattering (DLS) was measured with a Zetasizer Nano ZS (Malvern Instruments Ltd, UK) particle size analyzer. The temperatures changes were recorded by an infrared imager.

3. Synthesis of Cy-Cl

Bromide iodide salt (661.2 mg, 2 mmol) and 2-chloro-3-(hydroxymethylidene) cyclohexene-1-carbaldehyde (173.5 mg, 1 mmol) were dissolved in the mixture of 1-butanol (3 mL) and methylbenzene (7 mL). After stirring for 5 h at 115 °C, the mixture was precipitated into methyl tertiary butyl ether and filtered to afford green solid. The mixture was further purified by column chromatography (dichloromethane: methanol = 98:2) to afford Cy-Cl as green solid (543.6 mg, yield 65%). ^{1}H NMR (400 MHz, DMSO) δ 8.27 (dd, J = 14.0, 8.1 Hz, 2H), 7.68 (d, J = 7.3 Hz, 2H), 7.42-7.34 (m, 8H), 7.4-7.28 (m, 8H) 6.42 (dd, J = 14.1, 5.5 Hz, 2H), 5.54 (s, 4H), 2.51 (s, 6H), 1.74 (s, 12H).

4. Synthesis of Cy-TPA

Cy-Cl (383 mg, 0.5 mmol) and 4-N,4-N-diphenylbenzene-1,4-diamine (260 mg, 1 mmol) were dissolved in DMF (10 mL). After stirring for 4 h at 80 °C, the mixture was extracted by dichloromethane/water and then the organic layer was dried with anhydrous Na_2SO_4 and evaporated to remove solvent to afford blue solid. The mixture was further purified by column chromatography (dichloromethane: methanol = 97:3) to afford Cy-TPA as blue solid (311.2 mg, yield 63%). 1H NMR (400 MHz, DMSO) δ 8.81 (s, 1H), 7.95 (d, J = 13.7 Hz, 2H), 7.56 (d, J = 7.4 Hz, 2H), 7.36-7.31 (m, 6H), 7.31-7.27 (m, 4H), 7.24 (dd, J = 7.7, 3.8 Hz, 6H), 7.21-7.17 (m, 4H), 6.96 (t, J = 5.9 Hz, 4H), 6.92 (d, J = 5.6 Hz, 2H), 6.89 (d, J = 7.9 Hz, 4H), 6.09 (d, J = 13.8 Hz, 2H), 5.38 (s, 4H), 2.44 (s, 4H), 1.74 (s, 2H), 1.45 (s, 12H). HRMS (ESI, m/z): Calculated for C62H59N4+: 859.4734, found 859.4710.

5. Synthesis of Cy-Ph

Cy-Cl (383 mg, 0.5 mmol) and aniline (93.1 mg, 1 mmol) were dissolved in DMF (10 mL). After stirring for 4 h at 80 °C, the mixture was extracted by dichloromethane/water and then the organic layer was dried with anhydrous Na_2SO_4 and evaporated to remove solvent to afford blue solid. The mixture was further purified by column chromatography (dichloromethane: methanol = 97:3) to afford Cy-Ph as blue solid (284.2 mg, yield 66%). 1H NMR (400 MHz, DMSO) δ 8.84 (d, J = 13.3 Hz, 1H), 7.96 (d, J = 13.8 Hz, 2H), 7.49 (d, J = 7.4 Hz, 2H), 7.29 (m, 17H), 7.14 (d, J = 6.9 Hz, 2H), 6.97 (d, J = 7.9 Hz, 2H), 6.08 (d, J = 13.8 Hz, 2H), 5.38 (s, 4H), 2.47 (d, J = 13.8 Hz, 4H), 1.76-1.71 (m, 2H), 1.35 (s, 12H).

6. Preparation of the Nanoparticles

Nano-dialysis method with DSPE-mPEG2000 was used to prepare nanoparticles of the Cy-TPA NPs and Cy-Cl NPs. A mixture of Cy-TPA or Cy-Cl (1.8 mg) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(poly(ethylene glycol))-2000] DSPE-$mPEG_{2000}$ (10 mg) were dissolved in dimethyl formamide (DMF, 1 mL) under stirring for 1 h. Then the mixture was added dropwise to distilled water (10 mL). The mixture was stirred for 24 h at room temperature. Cy-TPA NPs in solution was obtained by lyophilization.

7. Preparation of phosphate buffer solution

Different pH PBS (phosphate buffer solution) were prepared by using 50 mM potassium dihydrogen phosphate (for pH 3.0-6.0 buffer), and dipotassium hydrogenphosphate (for pH 6.1-9.0 buffer). The pH buffer solutions (for pH 1.0-4.2 buffer) was adjusted by adding 0.1 M HCl solutions.

8. The absorption and fluorescence spectra of molecules

The pH-dependent absorbance of Cy-TPA and Cy-Cl were obtained in buffer solution (DMSO: PBS, 1:99, v/v) with different pH (1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.2, 4.5, 5.0, 5.4, 5.8, 6.1, 6.4, 6.8, 7.0, 7.4). The pH-dependent absorbance of Cy-TPA NPs were obtained in PBS with different pH.

The pH-dependent fluorescent intensities of Cy-TPA and Cy-Cl at their maximal emission wavelength were obtained from the emission spectra in buffer solution (DMSO: PBS, 1:99, v/v) with different pH (4.2, 5.0, 5.4, 5.8, 6.1, 6.4, 6.8, 7.0, 7.4). The pH-dependent fluorescent intensities of Cy-TPA NPs were obtained from the emission spectra in PBS with different pH (4.2, 5.0, 5.4, 5.8, 6.1, 6.4, 6.8, 7.0, 7.4). The (pKa) of the probes were calculated based on Henderson-Hasselbalch treatment according to the fluorometric results.

as Cy-TPA NPs

$$log\left[\frac{I_{Fmax} - I_F}{I_F - I_{Fmin}}\right] = pH - pKa$$

I_F corresponds to the fluorescent intensity of measurements.

9. Fluorescence reversibility of Cy-TPA NPs

The pH of Cy-TPA NPs solution (10 μM) between pH 5.0 and pH 7.0 was adjusted back and forth by 2 M HCl or NaOH, and then measured by pH meter. The fluorescence spectra were recorded with λex = 801 nm.

10. Photothermal effect

The solutions (2 mL) containing different concentrations of Cy-TPA NPs (0, 5, 10, 15, 20, 40 μM) were irradiated by 660 nm laser. When the temperature reached a plateau, the irradiation was removed for cooling down to room temperature. The temperature of the solutions was recorded an interval of 20 s by an infrared thermal imaging camera.

11. Calculation of photothermal conversion efficiency

The photothermal conversion efficiency η is determined by equation:

$$\eta = \frac{hA(T_{max} - T_{surr}) - Q_{dis}}{I(1 - 10^{-A\lambda})}$$

Where η is the photothermal conversion efficiency, h is heat transfer coefficient, A is the surface area of the container, T_{max} is the highest temperature of the solution of Cy-TPA NPs reached, T_{surr} is the temperature of environment., I is the laser power, A_λ is the absorbance of Cy-TPA NPs, Q_{dis} is the heat dissipated from the light absorbed by the container.

12. pH-dependence of photothermal effect

A concentration of 20 μM Cy-TPA NPs in buffer solutions (2 mL) with pH increasing from 5.0 to 7.4 (5.0, 5.4, 6.4, 6.8, 7.4) were added to quartz cuvette. The solutions were irradiated by a laser (660 nm, 1.0 W/cm^2) for 18 minutes. The temperature of the solutions was recorded an interval of 1minutes by an infrared thermal imaging camera.

13. Photothermal stability

The solution of Cy-TPA NPs (20 μM) was placed in 2 mL quartz cuvette and irradiated by a laser (660 nm, 1.0 W/cm^2) for 10 minutes. After that, the laser was switched off, and the solution was cooled to room temperature naturally. This operation was repeated for five times and the maximum of fluorescence intensities at 801 nm were recorded.

14. Cell culture and cytotoxicity test

Murine breast cancer (4T1) cells were routinely seeded in DMEM with different pH (6.4, 6.8, 7.4) containing 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin (PS) at 37°C under a humidified atmosphere (5% CO_2).

CCK-8 assays were employed to detect the cell viabilities. 4T1 cells with different pH (6.4, 6.8, 7.4) were seeded in 96-well plates with a density of 1×10^5 cells per well at 37 ℃ for 4 h. Then the cells were incubated with Cy-TPA NPs at different concentrations (0, 1, 2, 5, 10, 25, 50, 100, 200 μM). After 24 h, The media were replaced by fresh culture media with CCK-8 reagent for an additional 3 h. The cell viabilities were measured by the OD value at 450 nm by a microplate reader.

15. Co-localization analysis of Cy-TPA NPs by CLSM

The 4T1 cells were treated with Cy-TPA NPs at a concentration of 20 μM for 3 h to ensure sufficient cellular uptake. Then, the cells were washed with PBS for three times and lysotracker green was used to stain the lysosome. Finally, the Cy-TPA NPs or lysotracker signal can be observed

from the emission wavelength in the range of 521-580 nm. The Cy-TPA NPs signal can be obtained with the emission from 650 to 750 nm.

16. Cell uptake of Cy-TPA NPs

4T1 cells with pH 6.4 were seeded in 96-well plates with a density of 1×10^5 cells per well at 37 ℃ for 4 h. Then the cells were incubated with Cy-TPA NPs at a concentration of 20 μM. After washing the cells with PBS for three times, the fluorescence signals of the Cy-TPA NPs were determined at different time points (0, 1,2 4, 8, 12, 18, 24 h) by the fluorescence microscope (EVOS f1, AMG).

17. PTT of Cy-TPA NPs *in vitro*

The 4T1 cells were incubated with the culture media with different pH (6.4, 6.8, 7.4) containing Cy-TPA NPs (1, 2, 5, 10 μM) for 12 h and then washed by PBS. The cells were under a laser irradiation (660 nm, 1 W/cm^2) for 10 minutes. After another 12 h, cell viability after PTT was investigated by CCK-8 assay.

18. Animals and tumor models

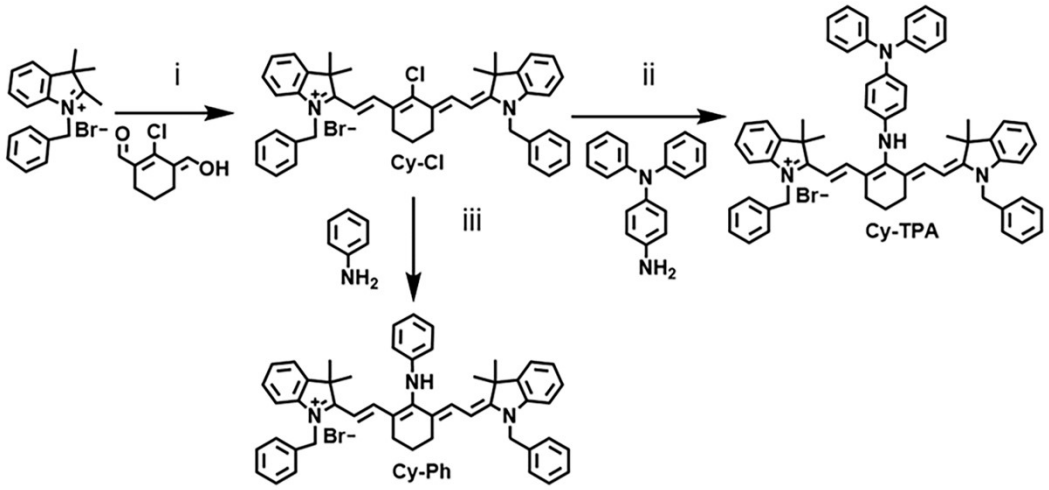
Female BALB/c mice (5 weeks old) were selected as the murine breast cancer (4T1) allograft tumor model according to Ethical Committee Peking Union Medical College and performed. Murine mammary carcinoma tumor models were established by subcutaneous injection of 4T1 cells in the right region of the mice back. The 4T1 tumor-bearing mice were selected for fluorescence imaging and photothermal therapy until the tumor volume reached 150 mm^3. The tumor volume (mm^3) was calculated as (tumor major axis) × (tumor minor axis) ^{2}/2.

19. Fluorescence imagining of Cy-TPA NPs *in vivo*

The 4T1 tumor-bearing mice were post intravenously (i.v.) injected with 200 μL of Cy-TPA NPs or Cy-Cl NPs (100 μM) respectively. In vivo fluorescence imaging was recorded on In vivo Imaging System (IVIS) Imaging Spectrum System and analyzed by an IVIS 3.0 Living Imaging software (PerkinElmer, U.S.). at different time points (0, 2, 4, 8, 12, 24, 36 and 48 h).

20. PTT of Cy-TPA NPs *in vivo*

The 4T1 tumor-bearing mice were post i.v. injected with 200 μL of Cy-TPA NPs (100 μM) respectively, and the tumors were irradiated by a 660 nm laser (1 W/cm^2) for 10 minutes at 12 h. Control groups were set as follows: Control 1, PBS; Control 2, only Cy-TPA NPs (100 μM, 200 μL); Control 3, only Cy-Cl NPs (100 μM, 200 μL); Control 4, only laser irradiation (660 nm, 1W/cm^2, 10 minutes); Cy-Cl NPs (100 μM, 200 μL) combining with laser irradiation (808 nm, 1W/cm^2, 10 minutes). The temperature of the mice was recorded by an infrared thermal imaging camera. After the treatment, the physiological information of mice was recorded every other day.



Scheme S1 Structure and synthesis of the probe Cy-TPA or Cy-Ph. Reagents and conditions: (i) n-butanol and toluene (7:3), reflux, 115 °C, 5 h. (ii) DMF, N,N-Diphenyl-p-phenylenediamine, 80 C, 4 h. (iii) DMF, aniline, 80 C, 4 h.

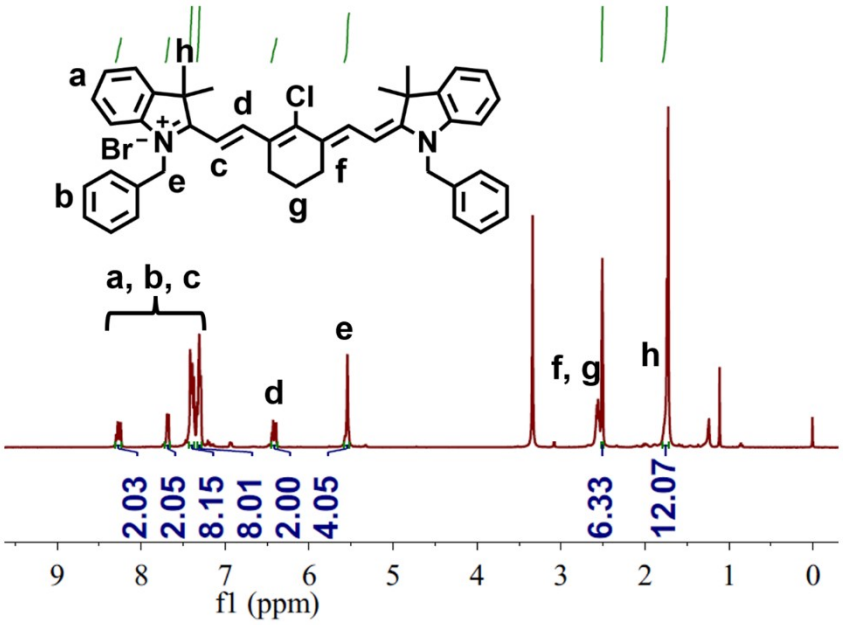


Fig. S1 ^{1}H NMR of Cy-Cl.

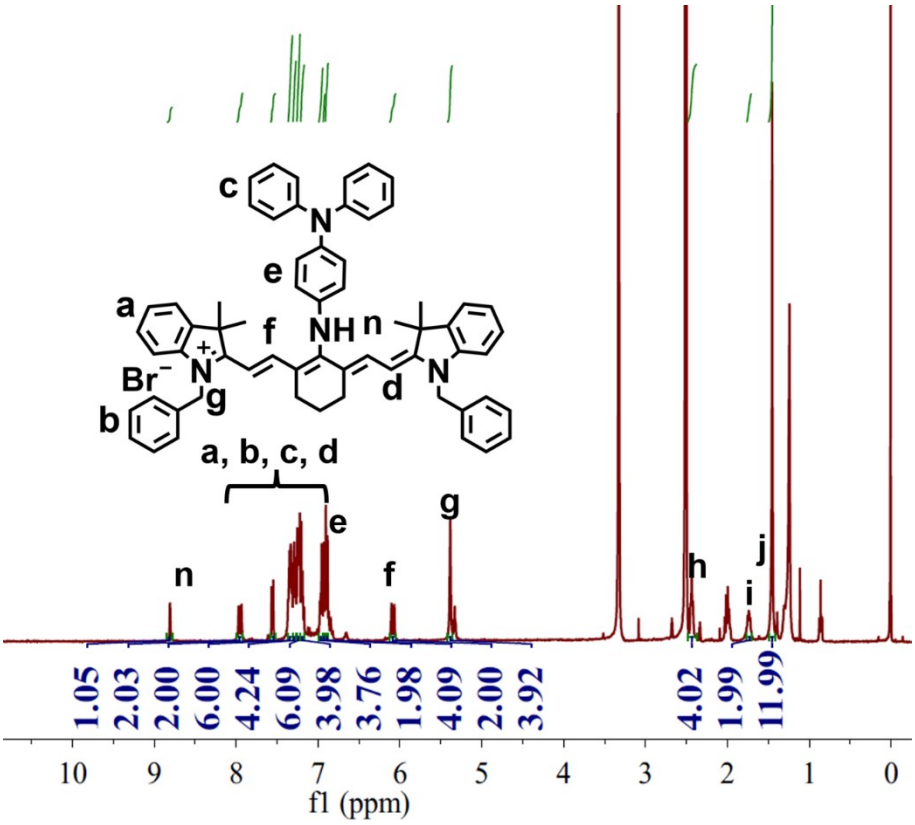


Fig. S2 ^{1}H NMR of Cy-TPA.

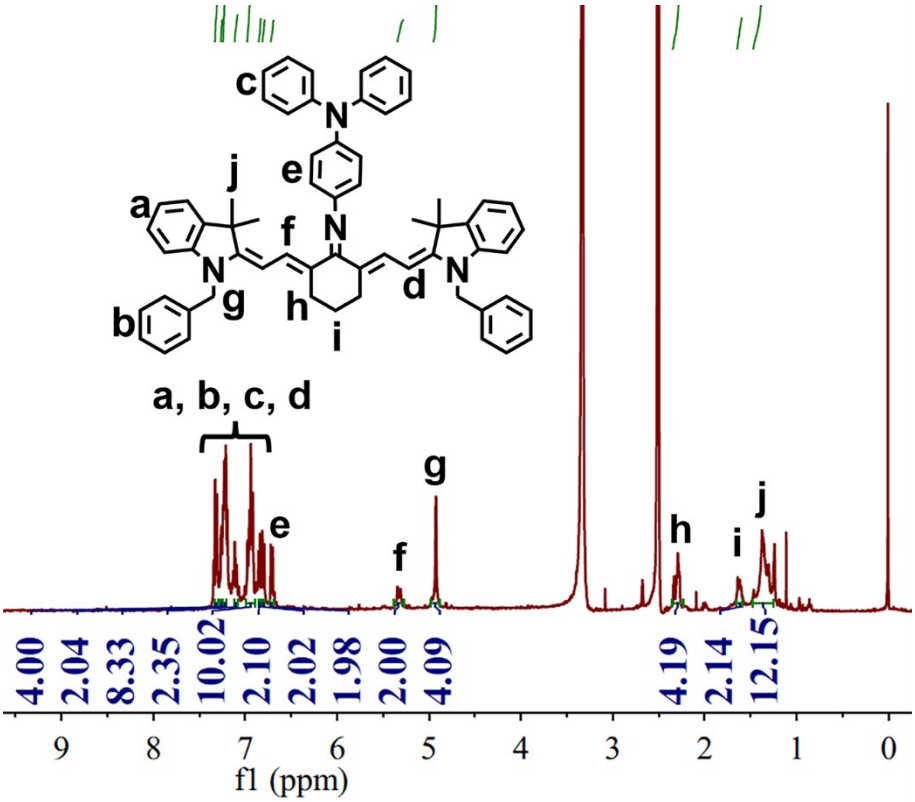


Fig. S3 ^{1}H NMR of Cy-TPA with potassium carbonate.

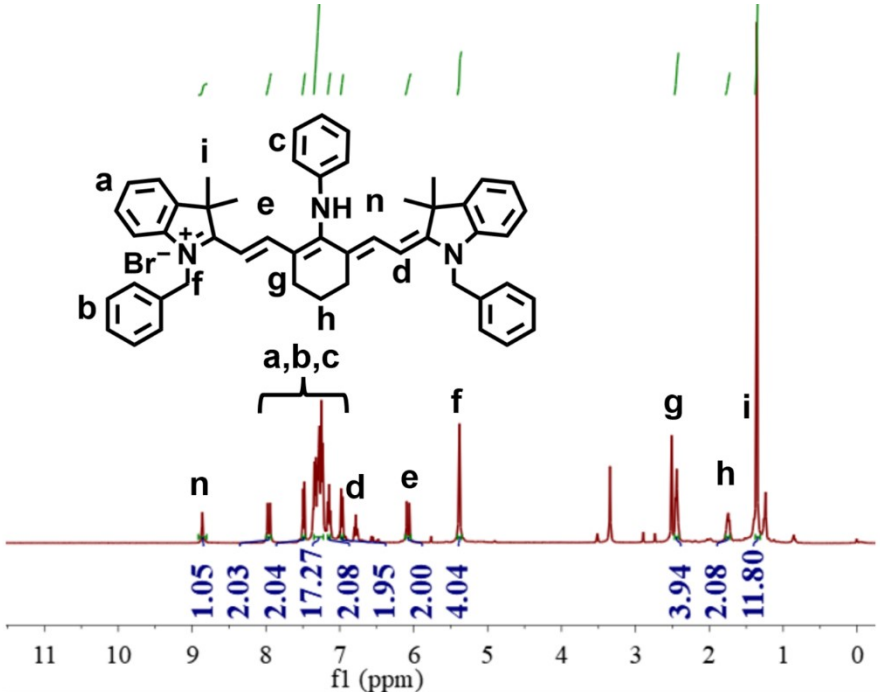


Fig. S4 ^{1}H NMR of Cy-Ph.

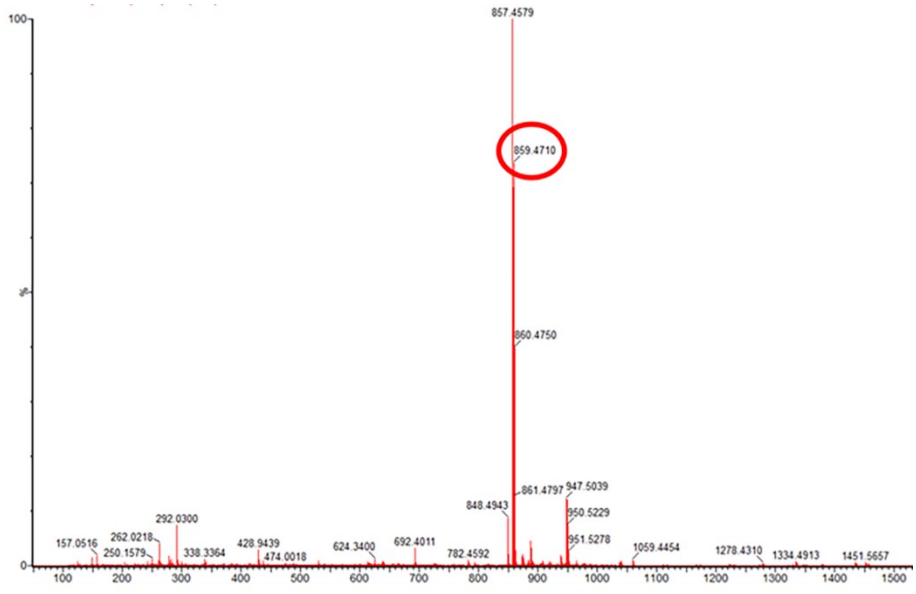


Fig. S5 Mass spectra of Cy-TPA.

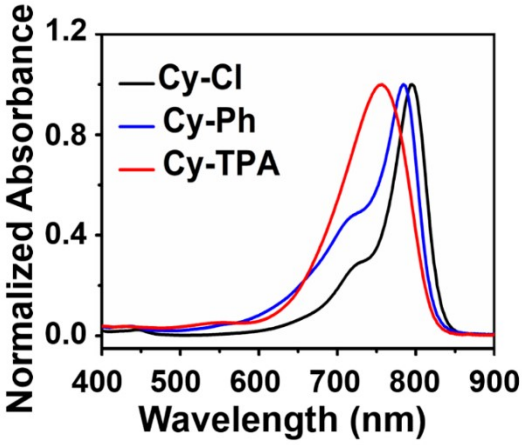


Fig. S6 The absorption spectra of 20 μM Cy-TPA, Cy-Ph and Cy-Cl in dichloromethane solution.

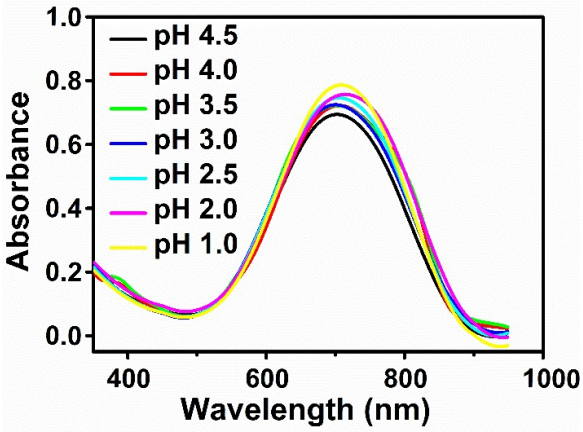


Fig. S7 The absorbance of 20 μM Cy-TPA in buffer solution (DMSO: PBS, 1:99, v/v) at different pH values (1.0-4.5).

The absorption spectra of Cy-TPA at pH range (1.0-4.5) show nearly no change of absorption peak at 725 nm, suggesting that the triphenylamine remains stable. Therefore, the nitrogen atom of the triphenyl amine moiety is not protonated upon lowering pH.

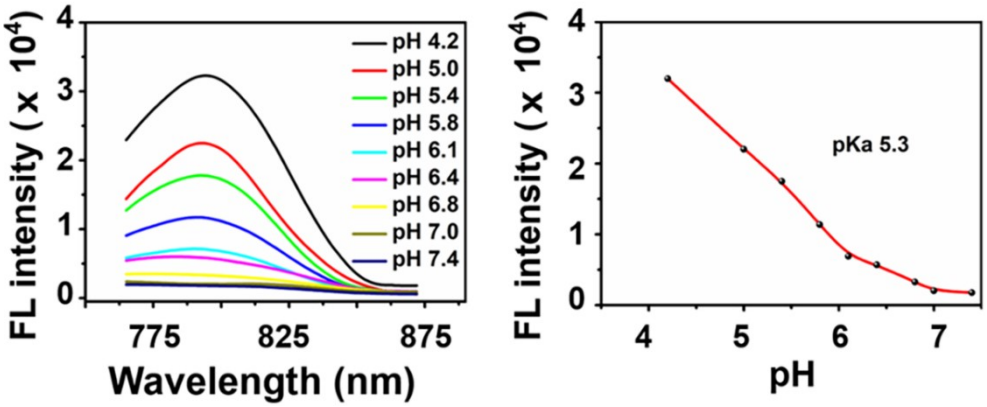


Fig. S8 The fluorescence spectra of 20 μM Cy-Ph in buffer solution (DMSO: PBS, 1:99, v/v) at different pH values (4.2-7.4).

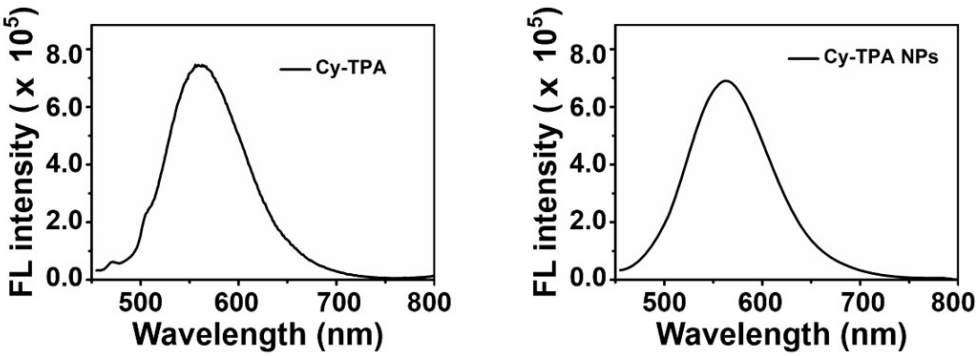


Fig. S9 The fluorescence spectra of 20 μM Cy-TPA in buffer solution (DMSO: PBS, 1:99, v/v) at pH 7.4 (left); the fluorescence spectra of 20 μM Cy-TPA NPs in PBS at pH 7.4 (right).

Under neutral condition, Cy-TPA as well as Cy-TPA NPs both exhibit a bright fluorescence at 560 nm, which is emit from the short-conjugated form. Therefore, pH-responsive mechanism of Cy-TPA should be the change of conjugation form, and should not be the protonation of nitrogen atom of triphenyl amine moiety.

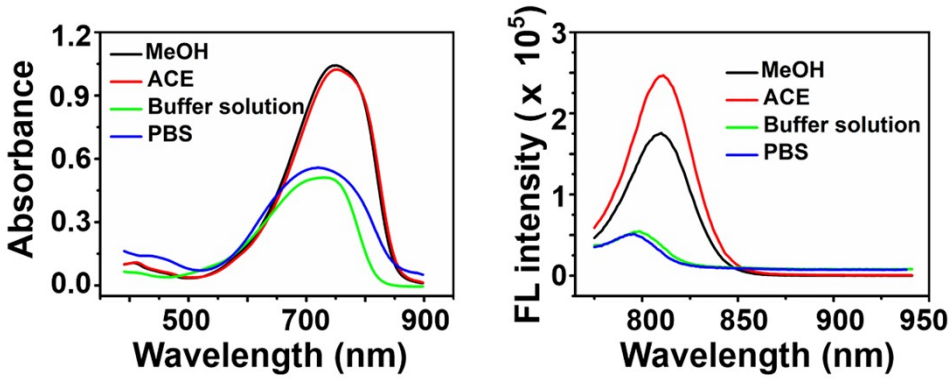


Fig. S10 The absorbance and fluorescence spectra of 20 μM Cy-TPA in methanol (MeOH), acetone (ACE), buffer solution (DMSO: PBS, 1:99, v/v) at pH 5.0 and 20 μM Cy-TPA NPs in PBS at pH 5.0.

Due to the effect of aggregation, the absorbance of Cy-TPA in buffer solution at pH 5.0 and Cy-TPA NPs in PBS at pH 5.0 are decreased compared to that of Cy-TPA in organic solvents. Besides, by the effect of aggregation, the fluorescence peak of Cy-TPA in buffer solution and Cy-TPA NPS in PBS appear at 797 and 801 nm respectively, which show blue-shifts compared to that of Cy-TPA in organic solvents (823 nm). Moreover, the fluorescence of Cy-TPA NPs in the PBS is decreased as Cy-TPA in buffer solution by the effect of aggregation. However, the flrorescence of Cy-TPA NPs is bright enough for imaging.

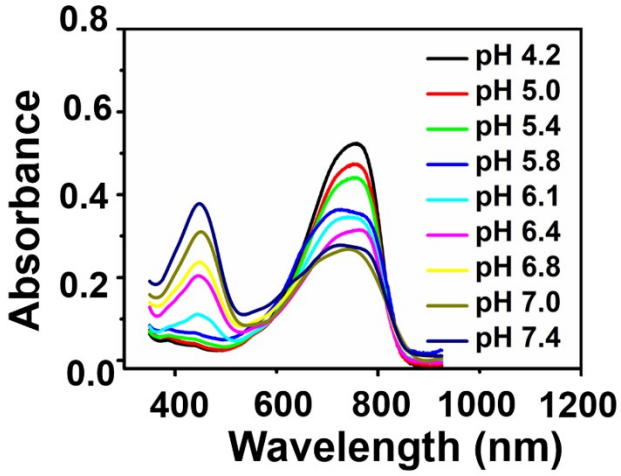


Fig. S11 The absorbance of 20 μM Cy-TPA NPs in PBS at different pH values (4.2-7.4).

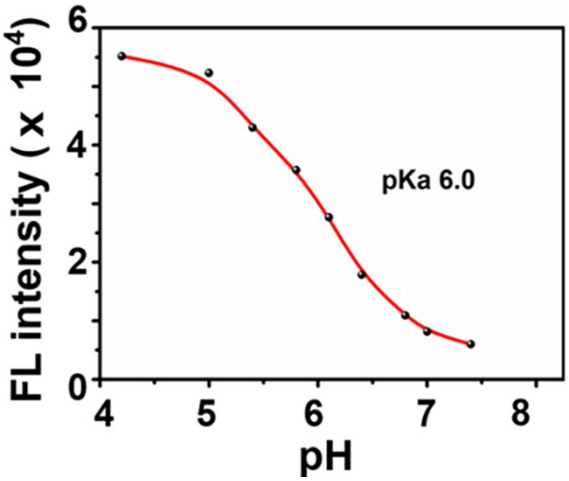


Fig. S12 The change of fluorescence intensities of 20 μM Cy-TPA NPs in PBS at different pH values (4.2-7.4).

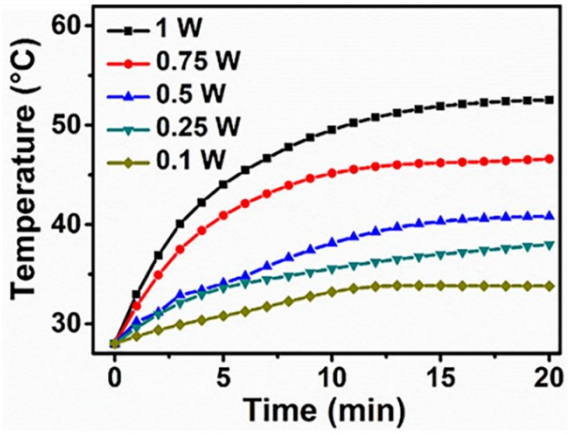


Fig. S13 Cy-TPA NPs (20 μM) under different laser densities (0.1-1.0 W/cm2) in buffer solution (pH 5.0).

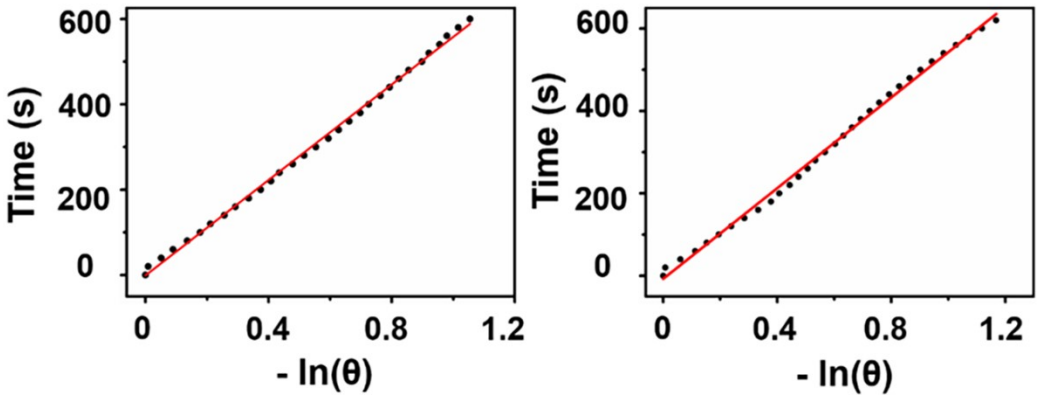


Fig. S14 Linear time data versus-ln (θ) (θ is the driving-force temperature) of Cy-TPA NPs (left) and Cy-Cl NPs (right). The $\tau_{\text{Cy-TPA NPs}}$ is 558 and $\tau_{\text{Cy-Cl NPs}}$ is 461.

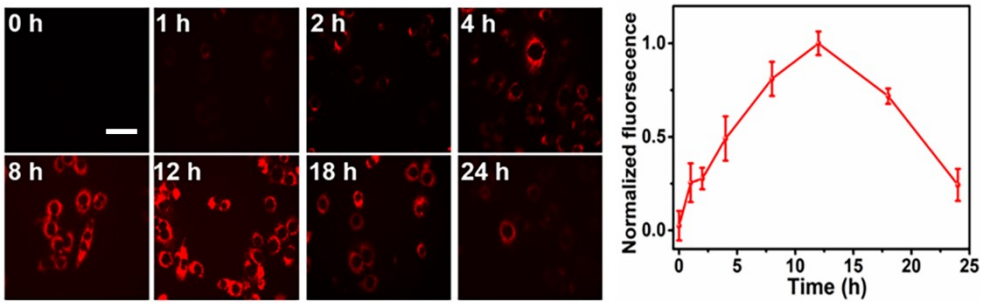


Fig. S15 Cellular uptake of Cy-TPA NPs (200 μM pH 6.4) at different time points. Scale bar = 200 μm.

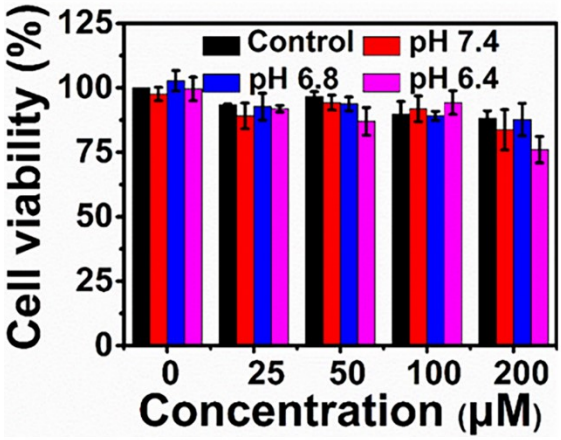


Fig. S16 Dark cytotoxicity of Cy-TPA NPs (0-200 μM, pH 6.4, 6.8, 7.4) in 4T1 cells.

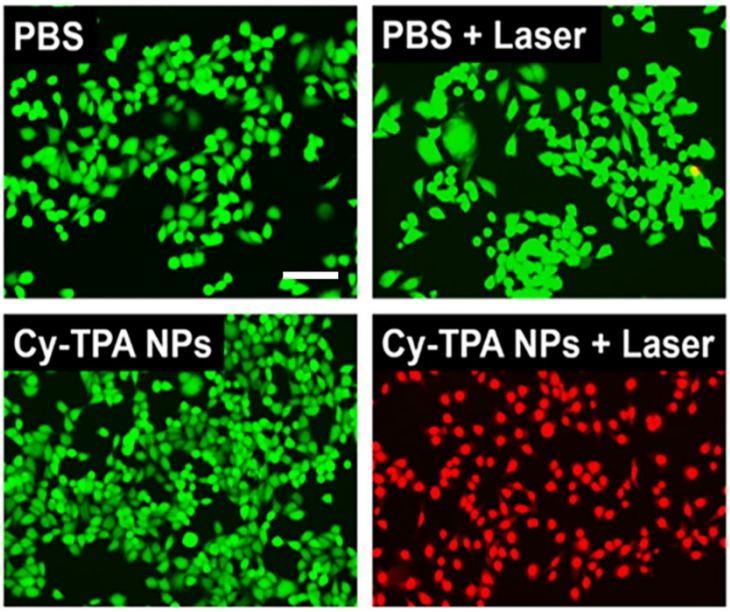


Fig. S17 Fluorescence imaging of Calcein AM (green, live cells) and Propidium iodide (red, dead cells) costained 4T1 cells treated with PBS, Cy-TPA NPs only, PBS + laser and Cy-TPA NPs + laser. Scale bar = 200 μm.

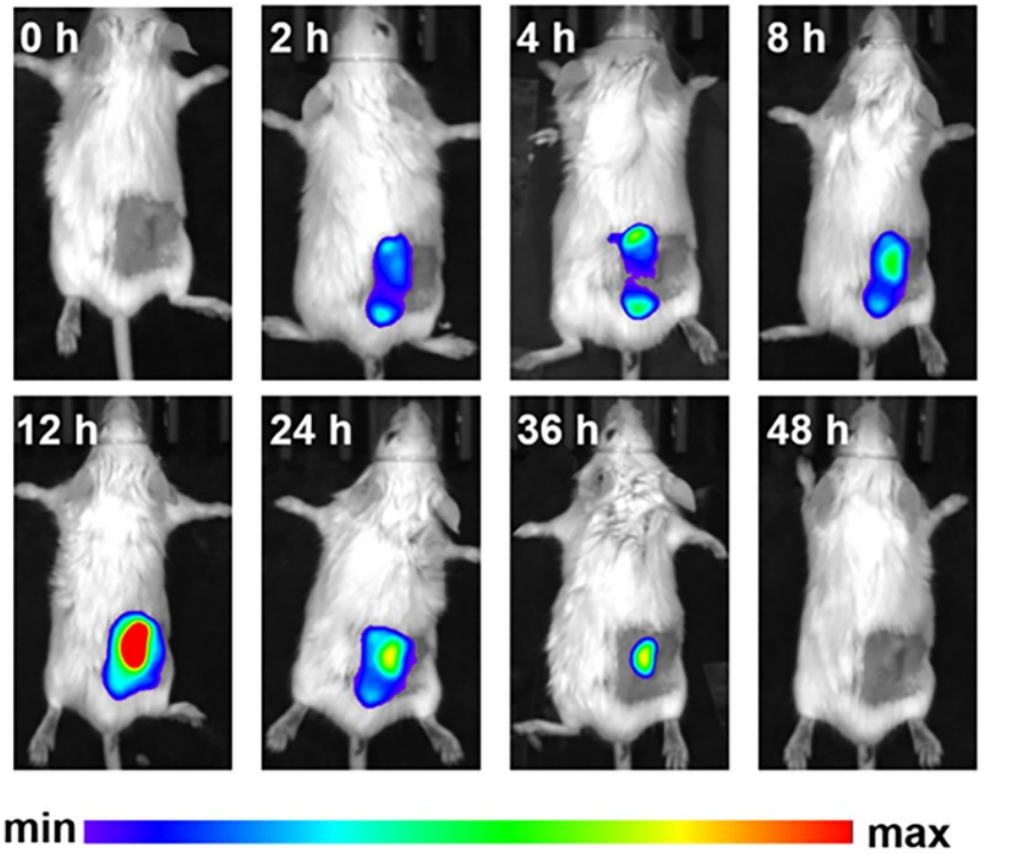


Fig. S18 Fluorescence imaging of 4T1 tumor-bearing mice after i.v. injection of Cy-Cl NPs at different time intervals.

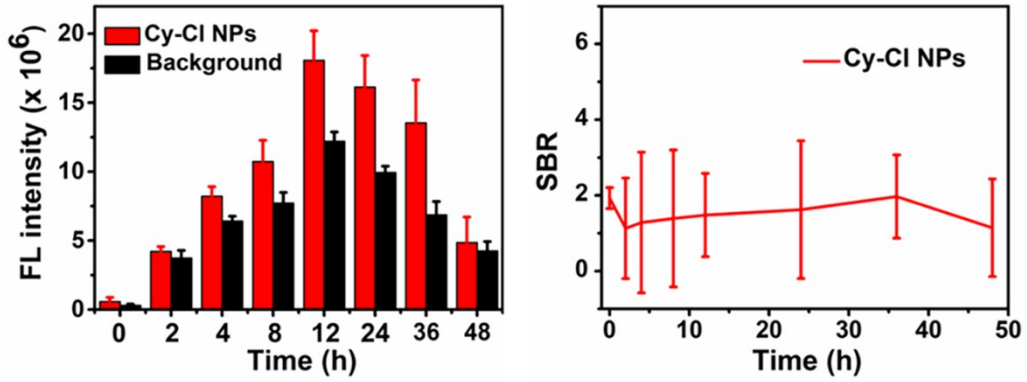


Fig. S19 Qualitative fluorescence intensity analysis of Cy-TPA NPs treated mice from Figure S18 (left) and the quantitative signal-background fluorescence intensity ratio of tumor (right).

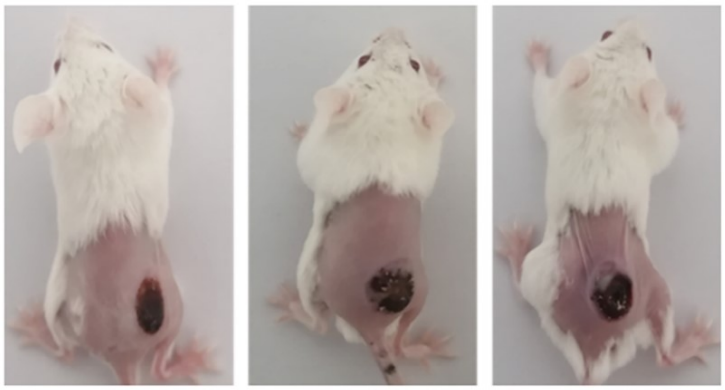


Fig. S20 Pictures of the mice treated with the Cy-TPA NPs (left), Cy-Cl NPs (middle) and PBS after laser irradiation (1.0 W/cm^2, 10 min).

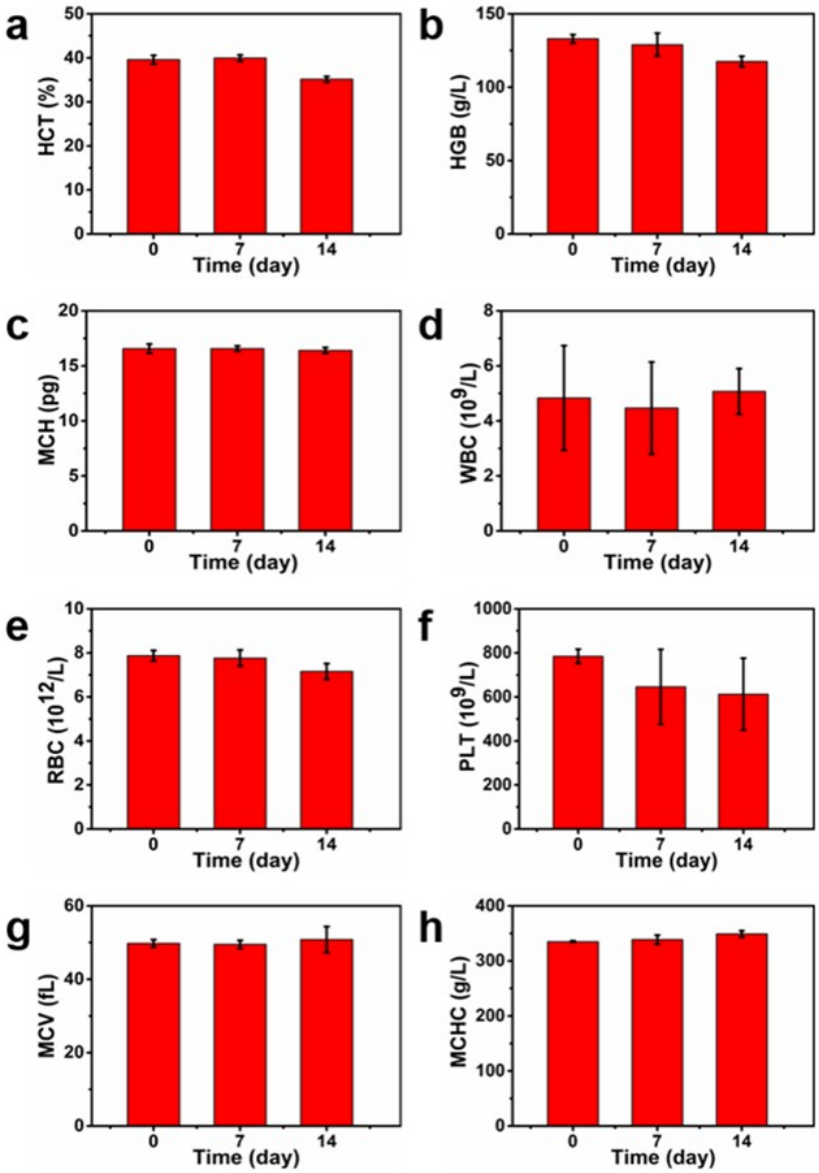


Fig. S21 Routine blood test analysis of mice after administrated with Cy-TPA NPs. (HCT: hematocrit, HGB: hemoglobin, MCH: mean corpuscular hemoglobin, WBC: white blood cell count, RBC: red blood cell count, PLT: platelet, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration).

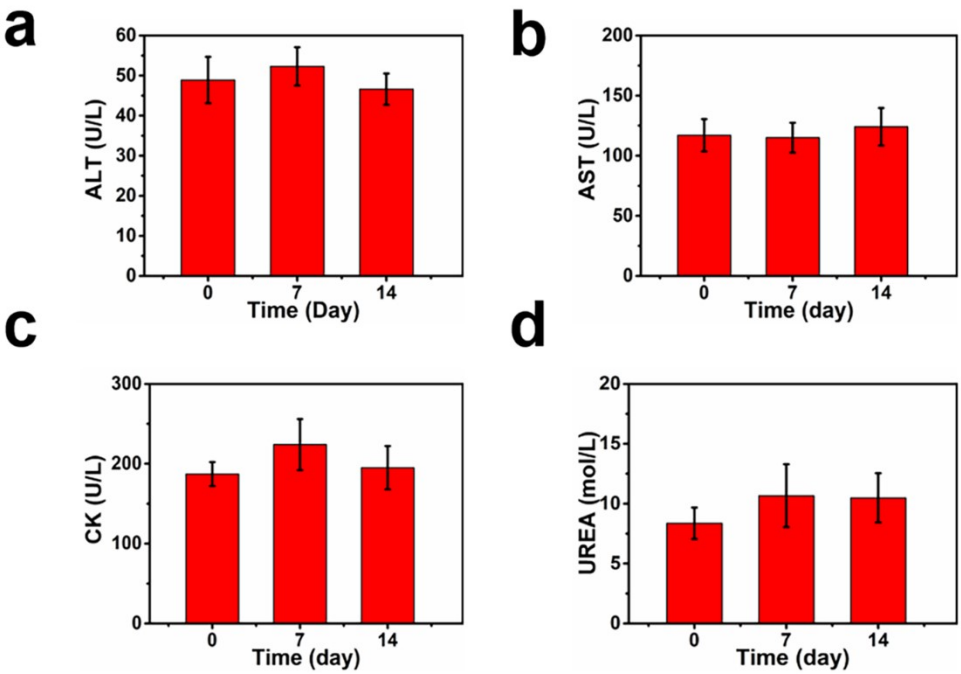


Fig. S22 Blood biochemical assay of mice after administrated with Cy-TPA NPs (ALT: alanine aminotransferase, AST: aspartate aminotransferase, CK: Creatine kinase, UREA: Urea).

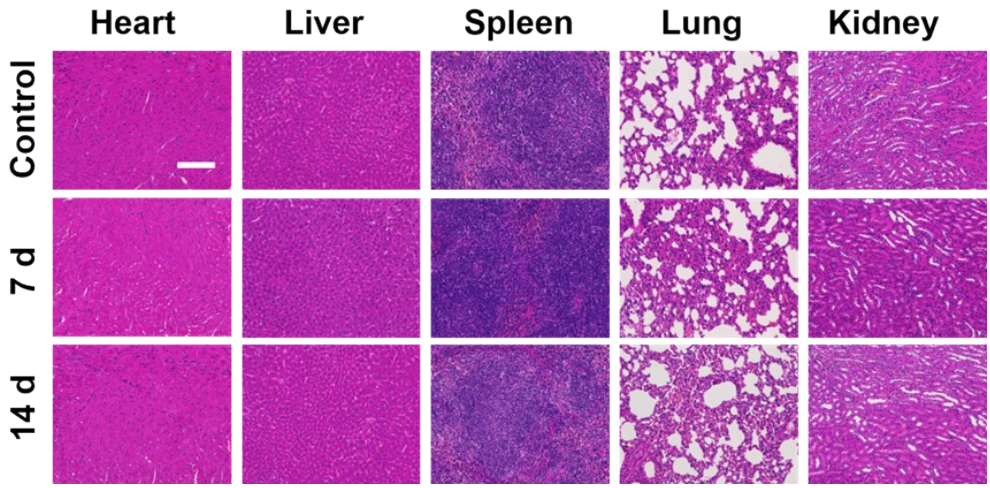


Fig. S23 H&E images of major organs (heart, liver, spleen, lung, and kidney) collected from different time points. Scale bar = 150 μm.