Hydrophilic Polyphosphoester-Conjugated Fluorinated Chlorin as an Entirely Biodegradable Nano-photosensitizer for Reliable and Efficient Photodynamic Therapy

Zhiyong Liu, Mengsi Wu, Yudong Xue, Chao Chen, Frederik R. Wurm*, Minbo Lan and Weian Zhang*

Materials

Pentafluoro benzaldehyde and pyrrole were purchased from Macklin Reagents (Shanghai). *p*-Toluenesulfonyl hydrazide was purchased from J&K Scientific Ltd (Shanghai, China). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 4-dimethylaminopyridine (DMAP) were obtained from Aladdin Reagents of China. Dichloromethane (DCM) and *N*, *N*-dimethylformamide (DMF) were dried over with calcium hydride (CaH_2) and distilled before use. All other chemicals were utilized as received without further purification.

Measurements

^1H NMR spectra in $CDCl_3$ were determined by a Bruker AVANCE III HD 400 Spectrometer with tetramethylsilane as the internal standard. The UV-Vis spectra of the samples were measured over different irradiation time intervals using a Thermo Scientific Evolution 220 spectrophotometer. Dynamic light scattering was performed on a BECKMAN COULTER Delasa Nano C particle analyzer at room temperature. TEM samples were prepared by dropping the nanoparticles solution (1 mg/mL) on to a carbon coated copper grid, and the images were observed on a JEOL JEM1400 electron microscope operated at 100 kV.

Methods

TFPC and $PMEP_{4000}$ were synthesized according to previous literatures.[1,2] Synthesis routines of **PPE-FP$_2$** and **PEG-FP$_2$** were shown in **Schemes S1-S2**.

Synthesis of TFPP

Pyrrole (268 mg, 4 mmol) and pentafluoro benzaldehyde (784.3 mg, 4 mmol) were dissolved in 300 mL dry dichloromethane in a 500 mL flask and then degassed with nitrogen for 30 min. Activated molecular sieve (10 mL) and boron trifluoride etherate (47% solution in diethyl ether, 0.3 mL, 1.12 mmol) were added subsequently. The mixture was shielded from ambient light. After stirring at room temperature for

4 h, 1 g (4.06 mmol) of *p*-tetrachlorobenzoquinone was added to the solution and the mixture was heated to reflux at 50 ℃ for 1 h. Then the solution containing the product was obtained by filtration under reduced pressure and concentrated by rotary evaporation. The crude product was further purified by column chromatography (dichloromethane: petroleum ether = 1:4) to obtain TFPP (400 mg, yield: 41%).

Synthesis of TFPP-COOH

TFPP (100 mg, 0.1 mmol) was dissolved in mixed solution of tetrahydrofuran (2 mL) and dimethyl sulfoxide (2 mL), 13 μL (0.095 mmol) of *tert*-butyl glycinate and 138 mg of potassium carbonate were added into the above solution. The mixture was stirred at room temperature for 6 h and then diluted by dichloromethane and washed with deionized water. The obtained organic layer was dried by anhydrous sodium sulfate and then evaporated to obtain dry powder. The mixture was further purified by column chromatography (dichloromethane: petroleum ether = 1:1). The obtained product was further reacted with trifluoroacetic acid to remove the *tert*-butyl group. TFPP-COOH was collected after the solvent was evaporated.

Synthesis of TFPC-COOH

TFPP-COOH (200 mg) and 1 g of *p*-toluenesulfonyl hydrazide were carefully mixed and then kept under vacuum for 1 h. Subsequently, the mixture was heated to 158 ℃ for 15 min. After cooling to room temperature, the mixture was dissolved in dichloromethane and then tetrachloro-*o*-benzoquinone (50 mg) was added into the solution. After the absorption of the mixture at 750 nm disappeared, the solution was washed with a saturated solution of sodium thiosulfate (2×), with distilled water (2×), and then dried over anhydrous Na_2SO_4. The TFPC-COOH was obtained after purified using silica gel chromatography (dichloromethane: methyl alcohol = 95 :5).

Synthesis of PPE-FP$_2$

TFPC-COOH (100 mg, 0.09 mmol) and 12.2 mg (0.01 mmol) of 4-dimethylaminopyridine were dissolved in dry dimethyl formamide (5 mL) under a nitrogen atmosphere. Then 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (95 mg, 0.48 mmol) was added into the mixture after PMEP (100 mg, 0.025 mmol) added and the mixture was stirred at room temperature for 48 h. Then the solution was transferred to a dialysis bag (MWCO = 3500) and dialyzed for 72 h against water. Finally, the product-containing solution was frozen and lyophilized under vacuum. **PEG-FP$_2$** as the control was synthesized

in a similar approach of **PPE-FP$_2$** by replaying PPE with PEG (average molecular weight: 4000 g/mol).

Self-assembly of PPE-FP$_2$ and PEG-FP$_2$

PPE-FP$_2$ (5 mg) and **PEG-FP$_2$** (5 mg) were separately dissolved in 5 mL of dimethyl formamide. Under vigorous stirring, 1 mL of the mixed solution was added dropwise to deionized water (9 mL) at room temperature. After stirring for 1 h, the organic solvent was removed by dialysis against deionized water for 24 h using a dialysis membrane (MWCO = 3500). Cy7-labelled **PPE-FP$_2$** and **PEG-FP$_2$** nanoparticles were obtained by co-assembly of **PPE-FP$_2$** or **PEG-FP$_2$** with Cy7 at a mass ratio of 5:1, respectively.

Singlet Oxygen Production

As a singlet oxygen scavenger, 1,3-diphenylisobenzofuran (DPBF) was used to determine the singlet oxygen production of nanoparticles. A solution containing a fixed concentration **PPE-FP$_2$** and DPBF was added into a quartz cuvette and irradiated at 655 nm for 180 s. The $^1O_2$ generation of **PPE-FP$_2$** can be directly correlated with the decrease of the DPBF absorbance in the UV-vis spectrum, thus the absorbance of DPBF at 415 nm was measured every 10 s. **PEG-FP$_2$** at the same photosensitizer concentration was tested as a control.

Cell Culture

4T1 murine breast cancer cells were purchased from Shanghai Institute of Cells, Chinese Academy of Sciences and cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% antibiotics (penicillin and streptomycin) and 10% fetal bovine serum (FBS) in a humidified standard atmosphere of 5% CO_2 at 37 ℃. DMEM and FBS were purchased from Shanghai Maokang Biotechnology.

Intracellular ROS Generation Assay

The intracellular ROS generation was measured by using dichlorofluorescein diacetate (DCFH-DA) as a probe. The DCFH-DA could be oxidized into highly fluorescent DCF by ROS. Typically, 4T1 cells with a density of 1×10^5 containing complete DMEM media were incubated in 6-well plates for 24 h. Then, the 4T1 cells were chosen to incubate with **PPE-FP$_2$** or **PEG-FP$_2$** for 24 h with or without light irradiated (655 nm, 5 min, 100 mW·cm^{-2}). The group without any treatment was acted as the control. Then, the cells medium was replaced with the fresh DMEM containing DCFH-DA solution at the concentration of 10 ×

10^{-6} M at 37 ºC in dark. After treatment for 30 min the cells were observed via confocal laser scanning fluorescence microscope to evaluate the ROS generation. The excitation wavelength was 485 nm, and the emission wavelength was 525 nm for the DCF detection.

***In Vitro Degradation Assay of PPE*-FP_2**

PPE-FP_2 and **PEG-FP_2** with the same amount of TFPC were dispersed in PBS (pH 7.4) with or without ALP, kept in closed sample vials and maintained in a horizontal shaker at 37 ℃. At predetermined time intervals, **PPE-FP_2** and **PEG-FP_2** solution were centrifuged at 5,000 r.p.m. to remove precipitate. The supernatant of the sample for different degradation time (0–7 day) were determined by the UV spectrum.

Cellular Uptake Evaluation

The cellular uptake was determined by using confocal laser scanning microscopy (CLSM). 4T1 cells (2×10^4 cells/well) were seed on glass bottom cell culture dish for 24 h, and then the cells were treated with fresh medium containing **PPE-FP_2** or **PEG-FP_2** at the same concentration 50 μg/mL of chlorin for 24 h. After that, cells were washed carefully and then treated with 4% paraformaldehyde. Then the cells nuclei were stained with Hoechst for 3 min and washed three times with PBS. Finally, intracellular fluorescence of chlorin was observed by CLSM with excitation at 404 nm and emission at 660 nm.

Cytotoxicity Assay

The cytotoxicity of **PPE-FP_2** was evaluated by a standard MTT assay. 200 μL of 4T1 cell suspension (1×10^4 cells/mL) was seeded in a 96-well plate and then incubated for 24 h at 37 ℃. Different concentrations of **PPE-FP_2** and **PEG-FP_2** (chlorin concentration at 0-50 μg/mL) in fresh DMEM media were added into the wells and co-cultured for another 24 h. The cells were washed and irradiated with 655 nm laser (100 mW/cm^2) for 10 min. Before the media was replaced with 200 μL of MTT solution (0.5 mg/mL in DMEM) and cultured for 4 h, the cells were incubated for further 24 h. Finally, 150 μL of DMSO per well was added to replace the MTT solution and dissolve the formazan, and the absorbance value was recorded with a SpectraMax spectrometer at the wavelength of 492 nm. The in vitro dark cytotoxicity of nanoparticles was checked using the same procedure described above but without illumination.

Animals and Tumor Xenograft Model

All animal procedures were in accord with the guidelines of the Institutional Animal Care and Use

Committee experiments were approved by the Animal Ethics Committee of the East China University of Science & Technology. Tumor-bearing BALB/c mice were established and used for *in vivo* imaging: 4T1 cells (10^6 in 200 μL PBS) were subcutaneously injected into the mice, respectively. Once the tumors reached the required volume, the tumor-bearing mice were used for imaging or therapy. The tumor volumes (V) were measured by the length and width of tumors and calculated as $V = 0.5 \times (\text{tumor length}) \times (\text{tumor width})^2$. Relative tumor volume was defined as V/V_0 (V_0 was the tumor volume when the treatment was initiated).

In Vivo Fluorescence Imaging

For fluorescence imaging, 200 μL of **PPE-FP$_2$** or **PEG-FP$_2$** nanoparticles labeled by Cy7 (1 mg/mL of chlorin) were injected into tumor bearing mice through the tail vein. All the image acquisitions were performed with *in vivo* multispectral imaging system (Kodak FX) equipped with excitation bandpass filter at 510 nm and 750 nm and emission at 700 nm and 830 nm when the mice were anesthetized at 0.5 h, 2 h, 4 h, 6 h, 12 h, 24 h and 36 h post-injection.

Therapeutical Evaluation of PPE-FP$_2$ for in Vivo

The 4T1 tumor-bearing mice were chosen for therapeutical evaluation of the **PPE-FP$_2$**. After the tumor volumes of the 4T1 tumor-bearing mice reached about 100 mm^3, the mice were injected with different agents and divided into five groups (n = 4 for each group): (1) PBS (2) **PEG-FP$_2$**, (3) **PPE-FP$_2$**, (4) **PEG-FP$_2$ + L** and (5) **PPE-FP$_2$ + L** (chlorin concentration at 1 mg/kg). Mice in **PEG-FP$_2$ + L** and **PPE-FP$_2$ + L** groups were irradiated once with 655 nm laser for 10 min at a power density of 200 mW/cm^2. The tumor volumes were measured and calculated by vernier caliper every two days. The body weights of mice were recorded during the whole experiments. Then, the mice were sacrificed on the 18th day. The tumors were dissected and weighed. The dissected tumors of the groups (1) PBS (2) **PEG-FP$_2$**, (3) **PPE-FP$_2$**, (4) **PEG-FP$_2$ + L** and (5) **PPE-FP$_2$ + L** were embedded in paraffin and made as 4 μm slices by cryosection. Furthermore, the frozen slices were stained with H&E to further characterize the therapeutic effects. The slices were imaged under an inverted fluorescence microscope.

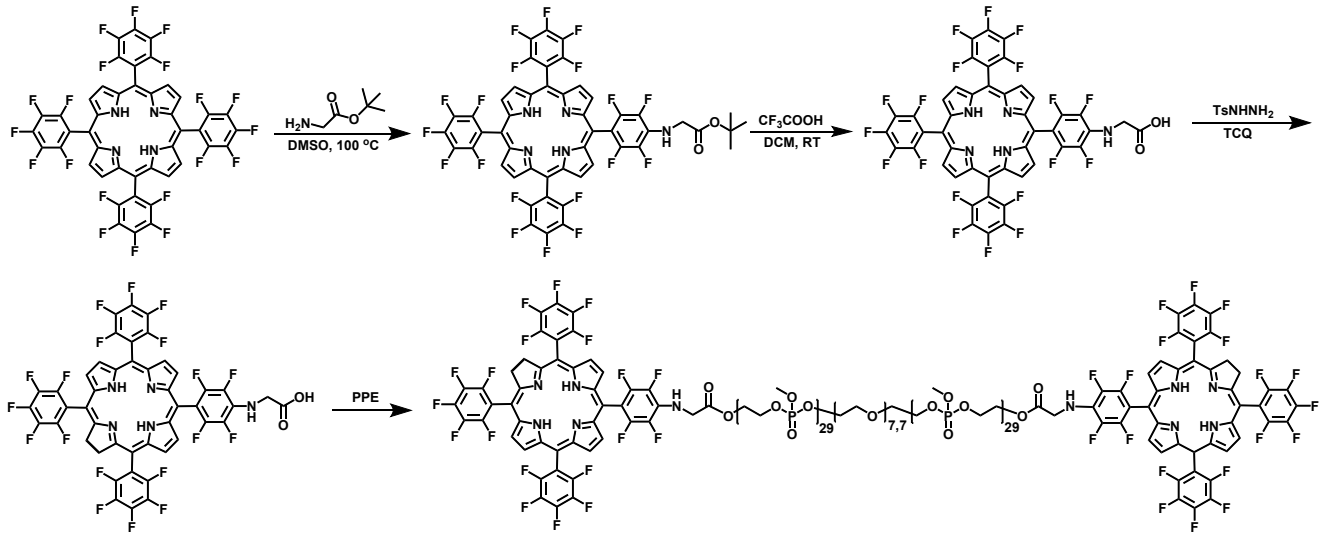
Toxicity Assessment of PPE-*FP$_2$ *and PEG-*FP$_2$ *in Vivo

To evaluate biological toxicity of nanoparticles *in vivo*, 200 μL of **PPE-FP$_2$** and **PEG-FP$_2$** at a dose of 10 mg/kg were intravenously (i.v.) injected into BALB/c mice (4-5 weeks old) through the tail vein twice

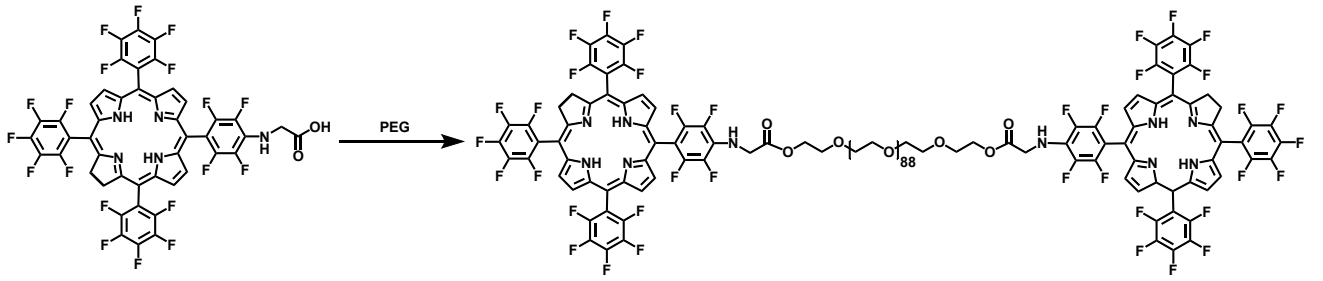
a week. Three animals were involved in each group. After i.v. injection of **PPE-FP$_2$** and **PEG-FP$_2$** for 60 days, the animals were euthanized. Liver, kidney and spleen were fixed in 10% neutral buffered formalin by immersion. Fixed tissues were dehydrated and embedded in paraffin. Tissues were sectioned and stained with hematoxylin and eosin (H&E), followed by microscopy examination.

References

[1] J. S. Lindsey, R. W. Wagner, *The J. Org. Chem.* **1989**, *54*, 828-836.
[2] C. Bernhard, K. N. Bauer, M. Bonn, F. R. Wurm, G. Gonella, *ACS Appl. Mater. Interfaces* **2018**, 11, 1624-1629.



Scheme S1. Synthesis of **PPE-FP$_2$**.



Scheme S2. Synthesis of **PEG-FP$_2$**.

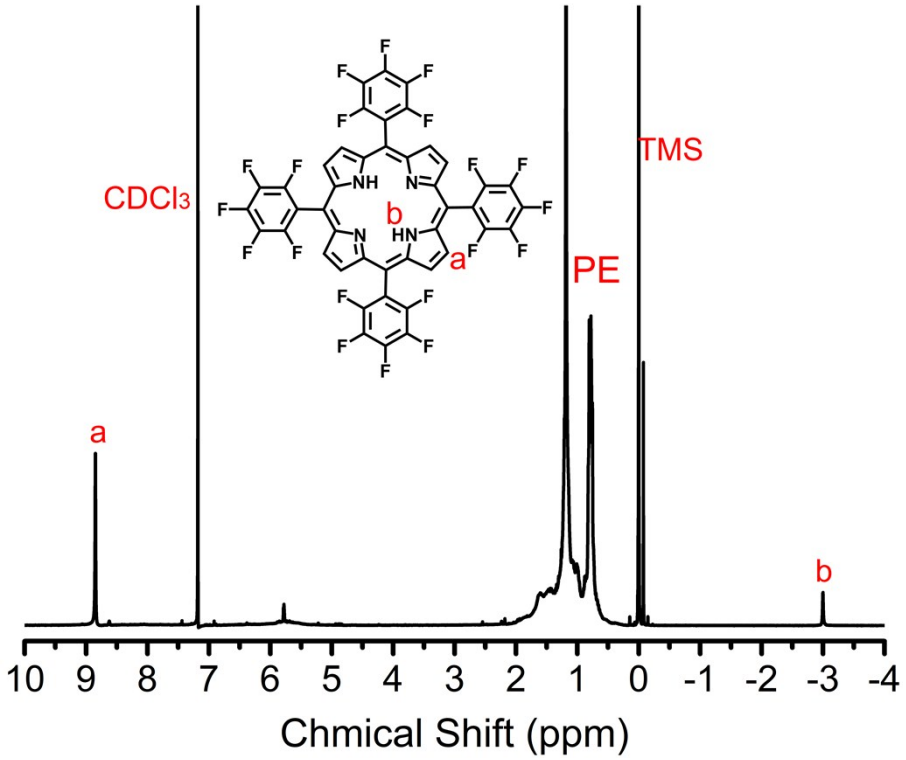


Fig. S1. ^{1}H NMR spectrum of TFPP.

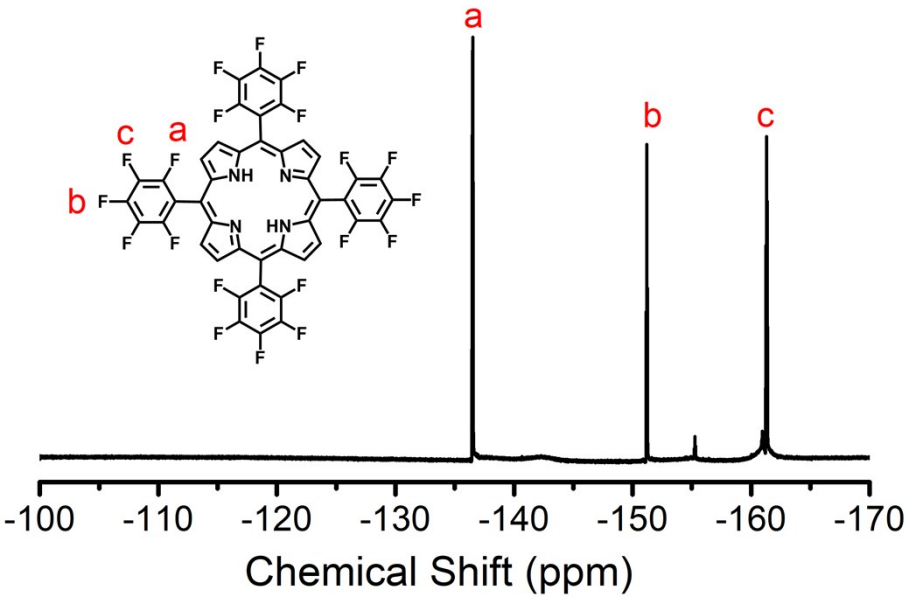


Fig. S2. ^{19}F NMR spectrum of TFPP.

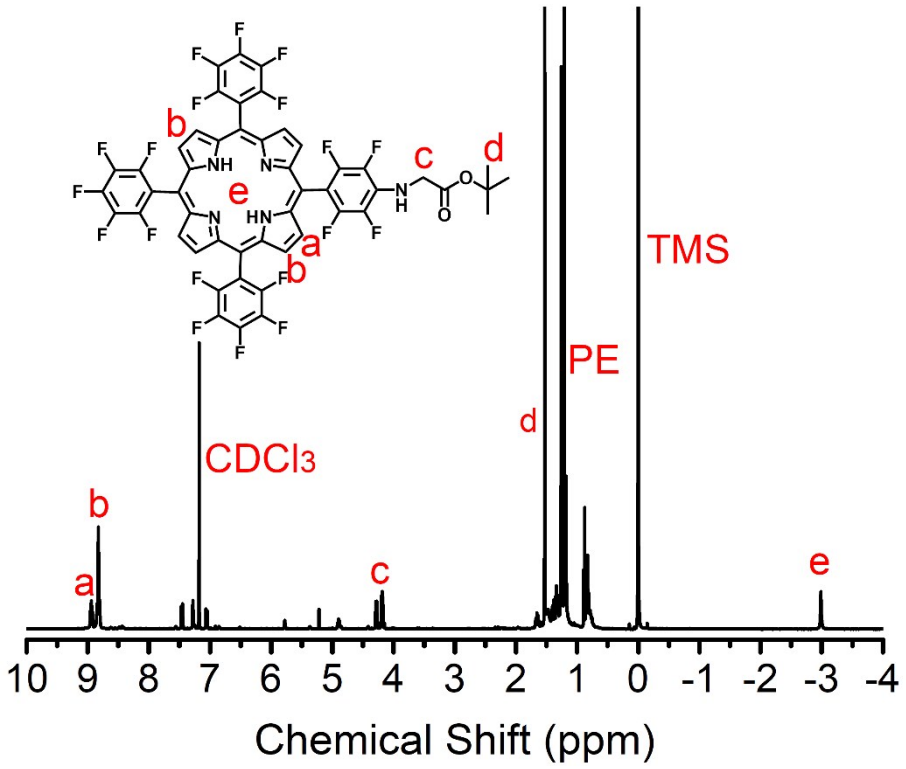


Fig. S3. [1]H NMR spectrum of TFPP-COO-*t*Bu.

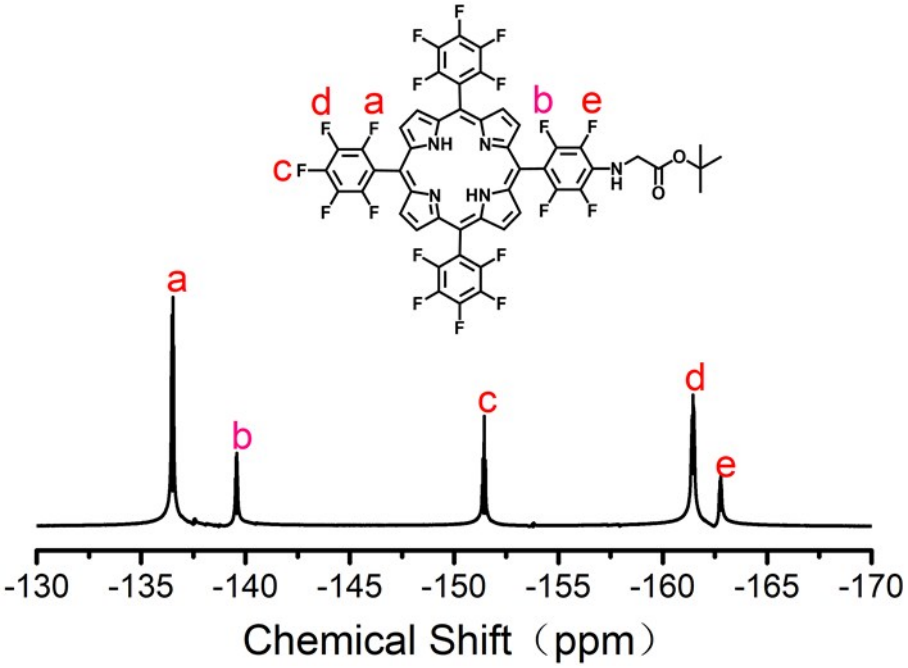


Fig. S4. [19]F NMR spectrum of TFPP-COO-*t*Bu.

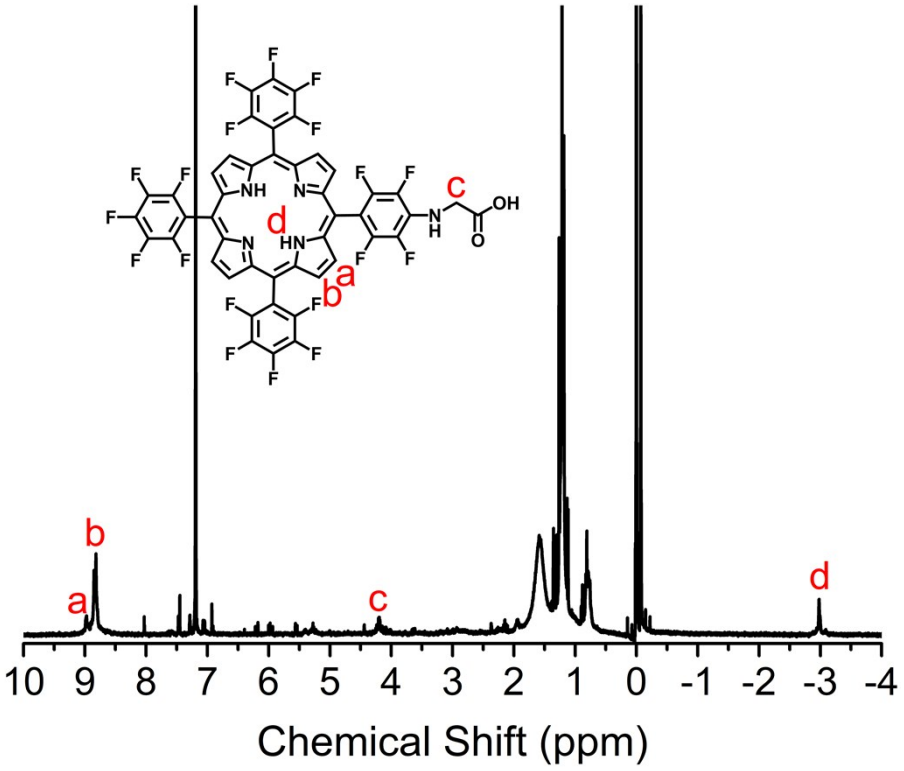


Fig. S5. ^{1}H NMR spectrum of TFPP-COOH

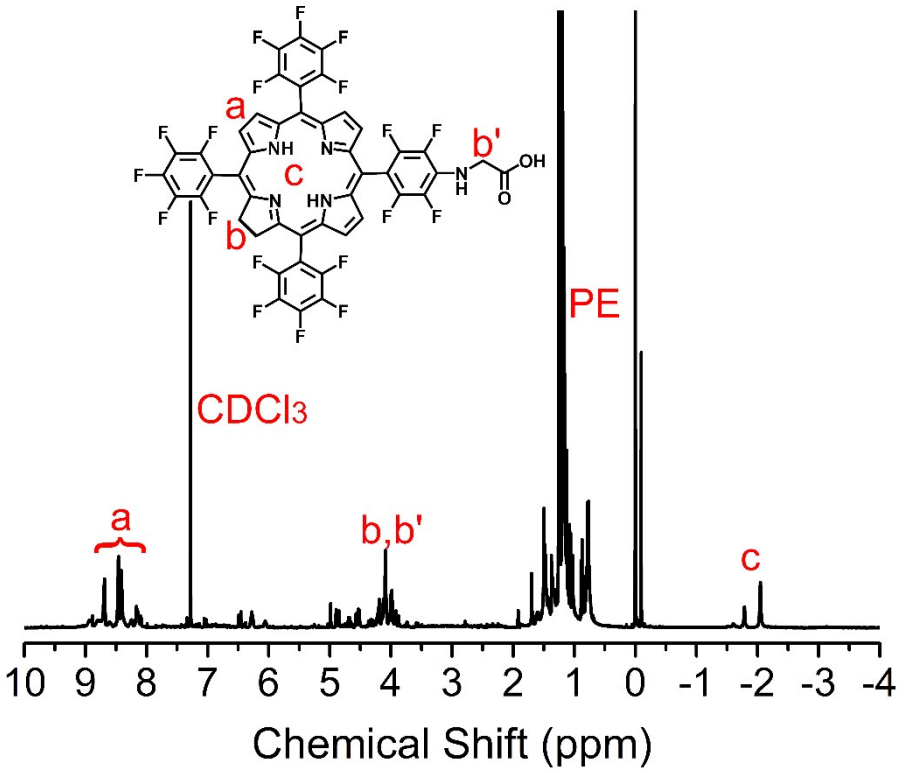


Fig. S6. ^{1}H NMR spectrum of TFPC-COOH.

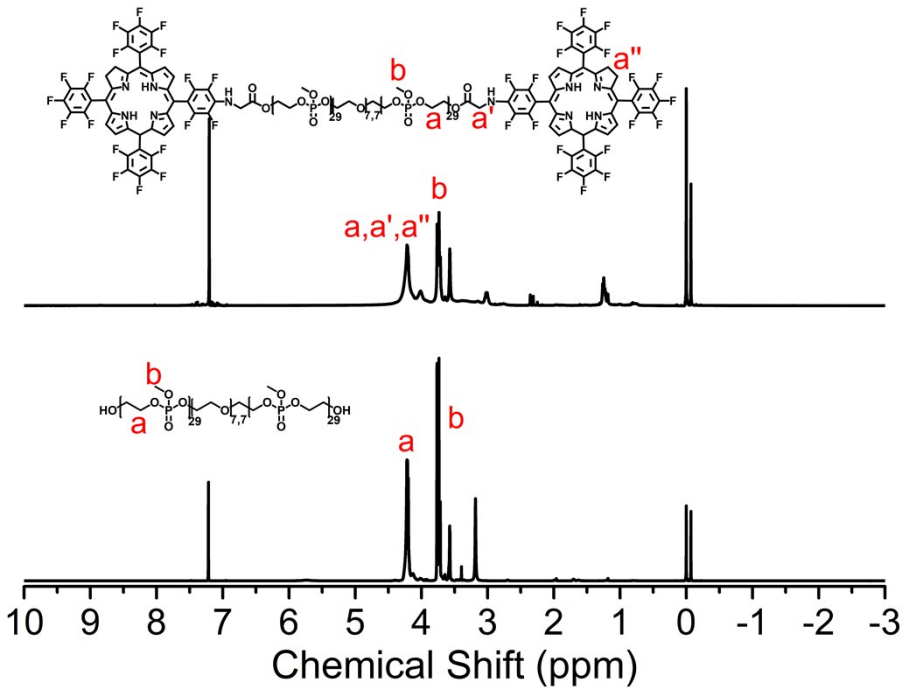


Fig. S7. ^{1}H NMR spectra of **PPE** and **PPE-FP$_2$** in $CDCl_3$.

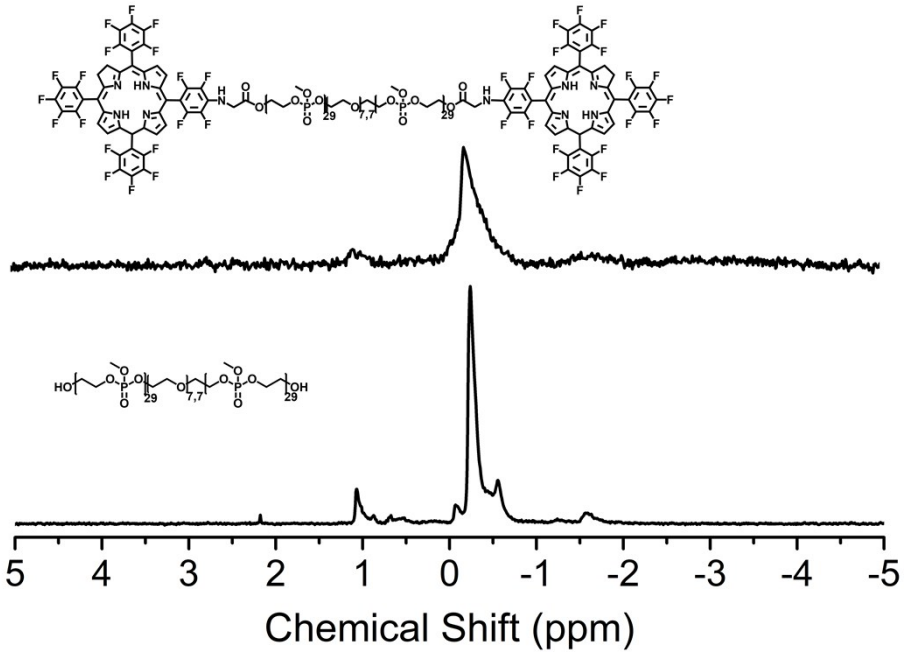


Fig. S8. ^{31}P NMR spectra of **PPE** and **PPE-FP$_2$** in $CDCl_3$.

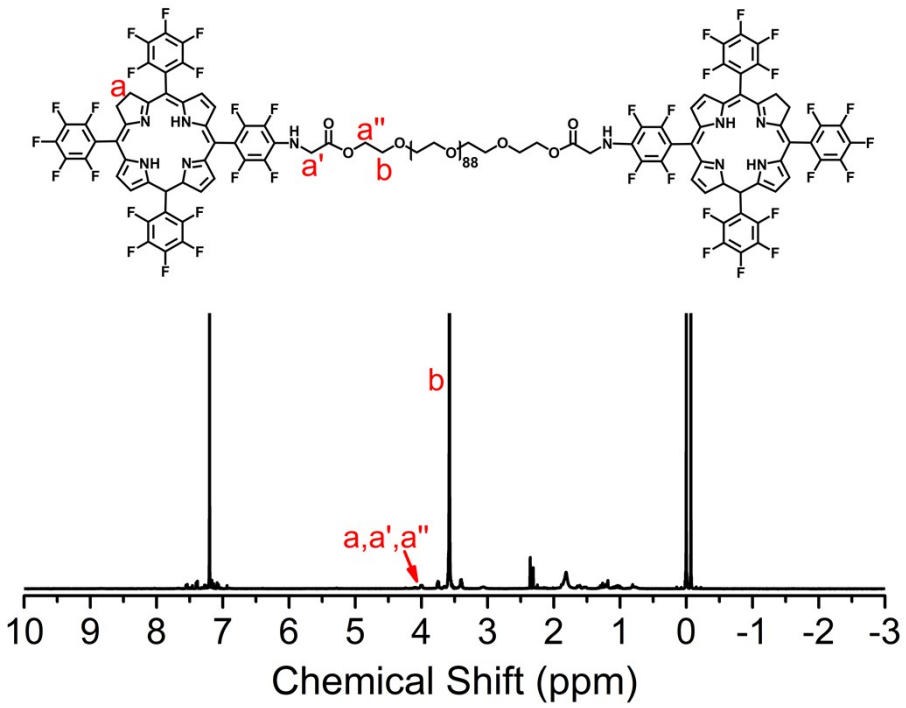


Fig. S9. ^{1}H NMR spectrum of **PEG-FP$_2$** in $CDCl_3$.

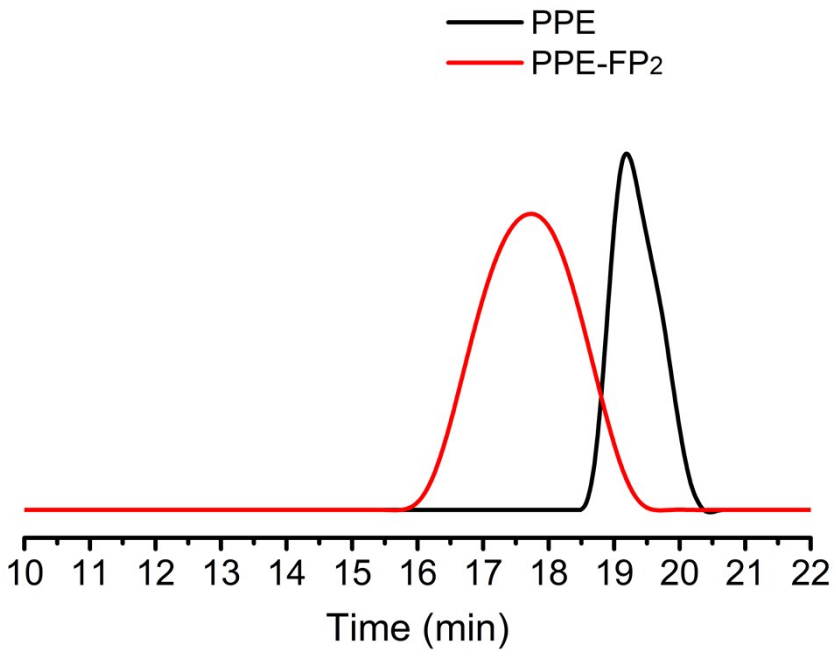


Fig. S10. GPC traces of PPE and **PPE-FP$_2$** in DMF.

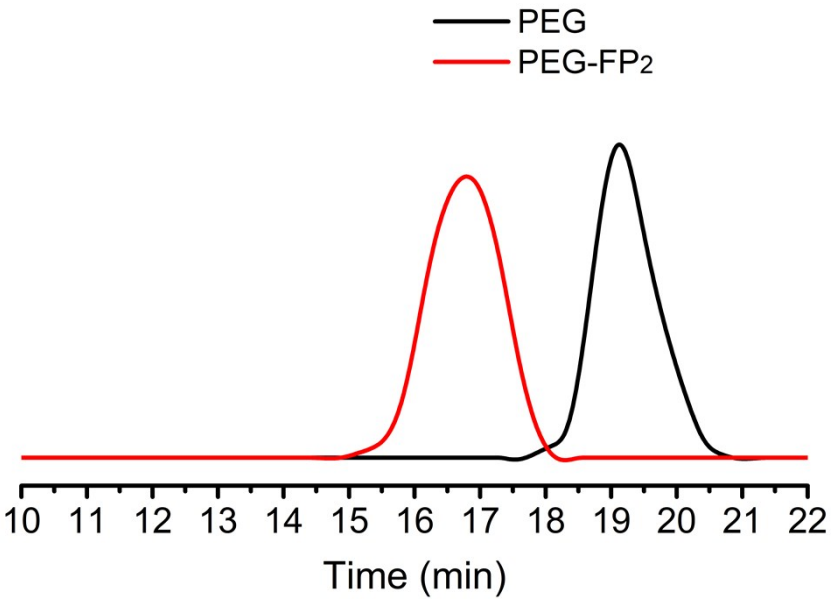


Fig. S11. GPC traces of PEG and **PEG-FP$_2$** in DMF.

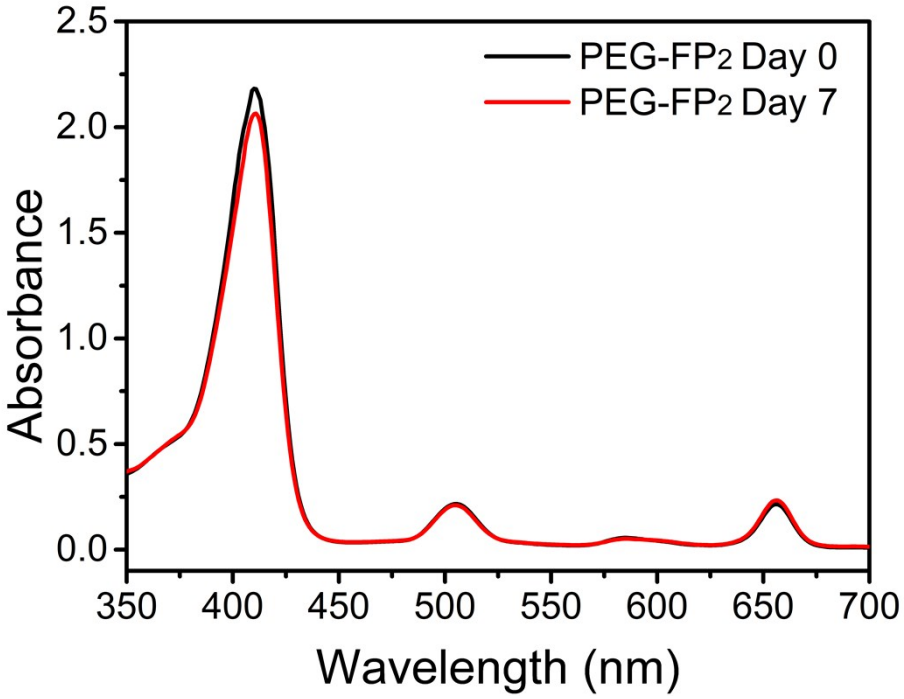


Fig. S12. UV spectra of **PEG-FP$_2$** at day 0 and day 7 in PBS (pH = 7.4).

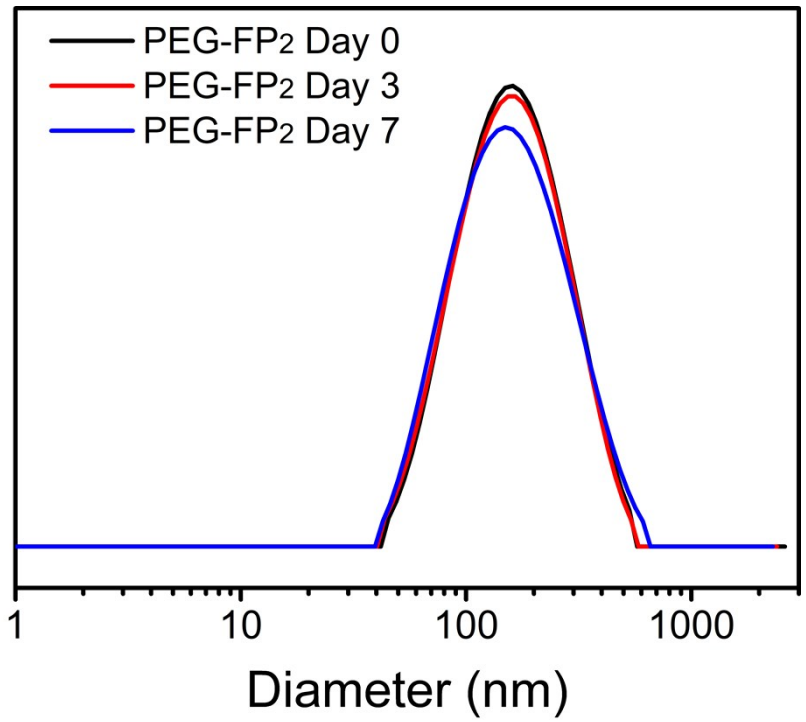


Fig. S13. Size distributions of **PEG-FP$_2$** determined by dynamic light scattering (PDI: day 0, 0.24; day 3, 0.23; and day 7, 0.22. Concentration: 0.5 mg/mL).

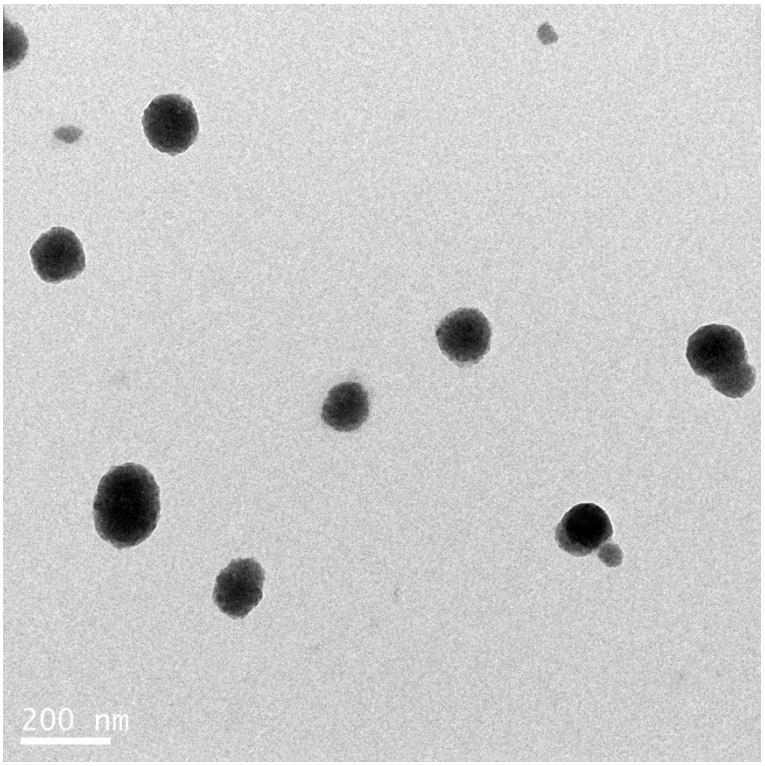


Fig. S14. TEM image of **PPE-FP$_2$** nanoparticles at day 1 in PBS (pH = 7.4).

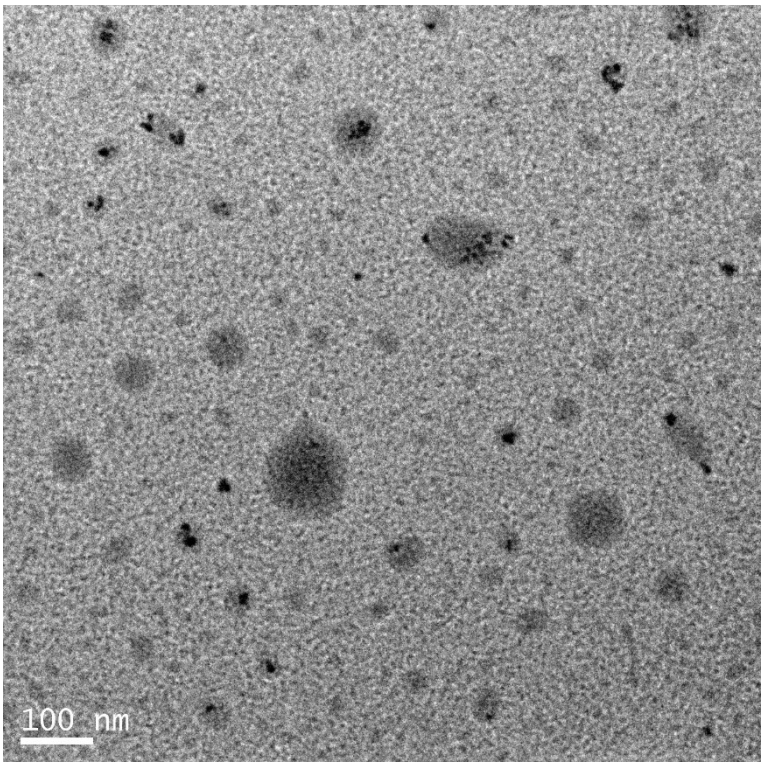


Fig. S15. TEM image of **PPE-FP$_2$** nanoparticles at day 3 in PBS (pH = 7.4).

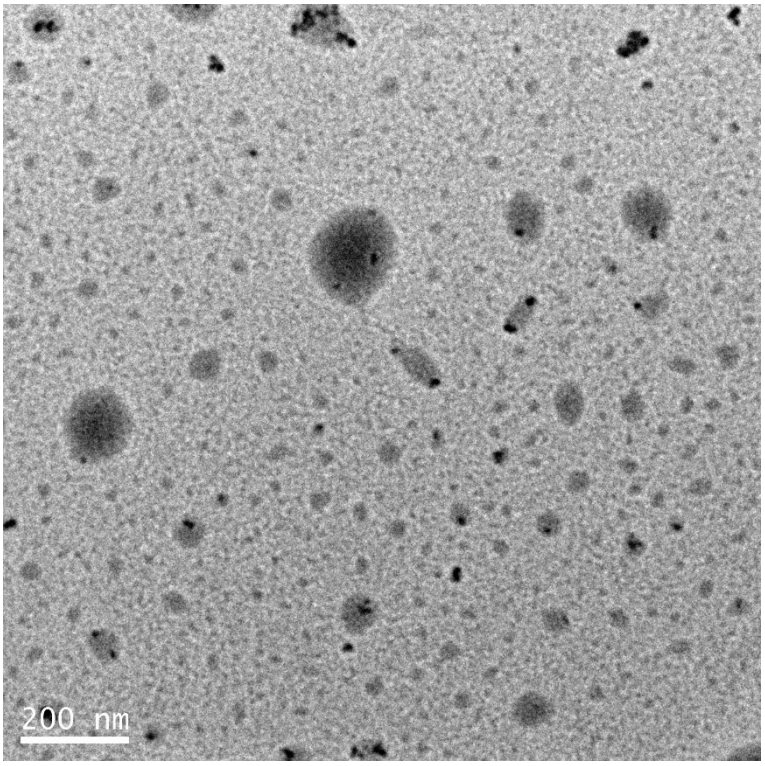


Fig. S16. TEM image of **PPE-FP$_2$** nanoparticles at day 1 in PBS with ALP (pH = 7.4).

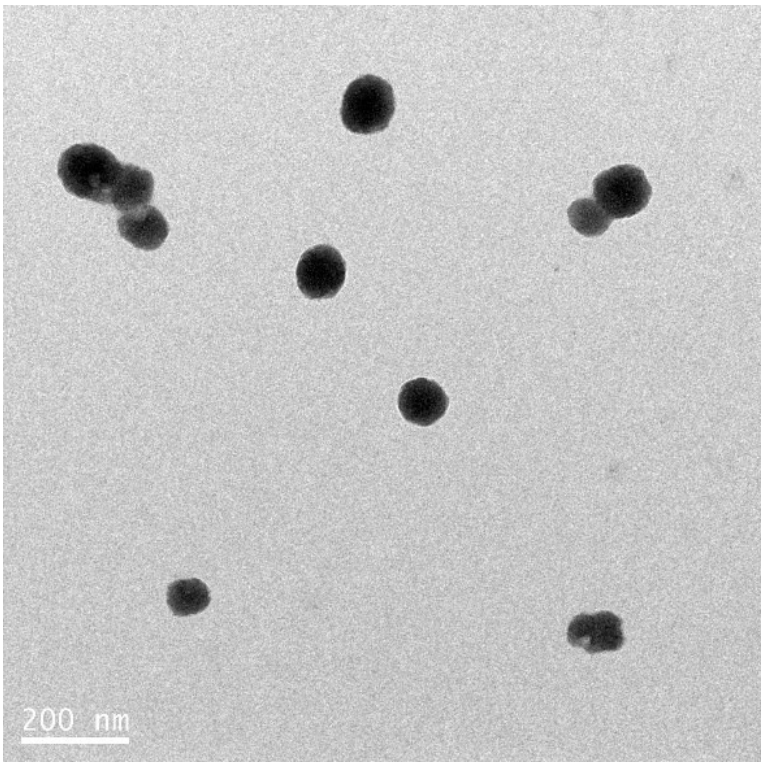


Fig. S17. TEM image of **PEG-FP$_2$** nanoparticles at day 7 in PBS (pH = 7.4).

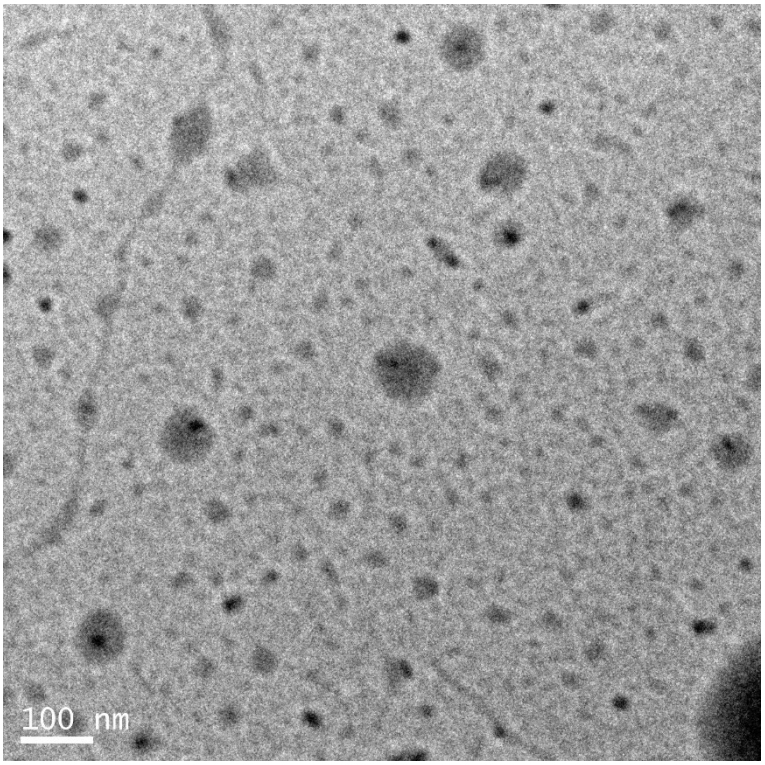


Fig. S18. TEM image of **PPE-FP$_2$** nanoparticles at day 7 in PBS (pH = 7.4).

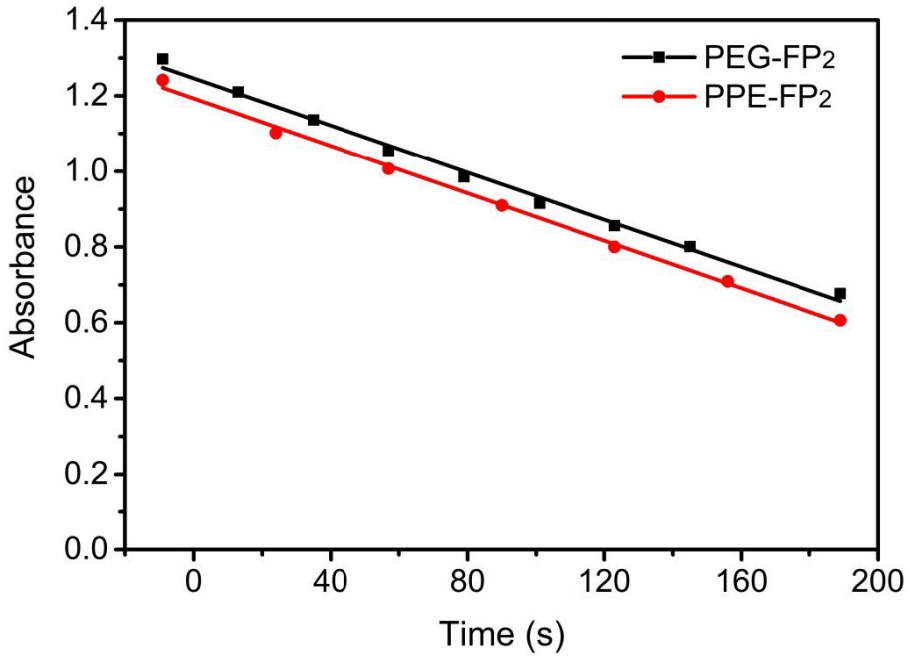


Fig. S19. ROS generation determined by DPBF as a probe.

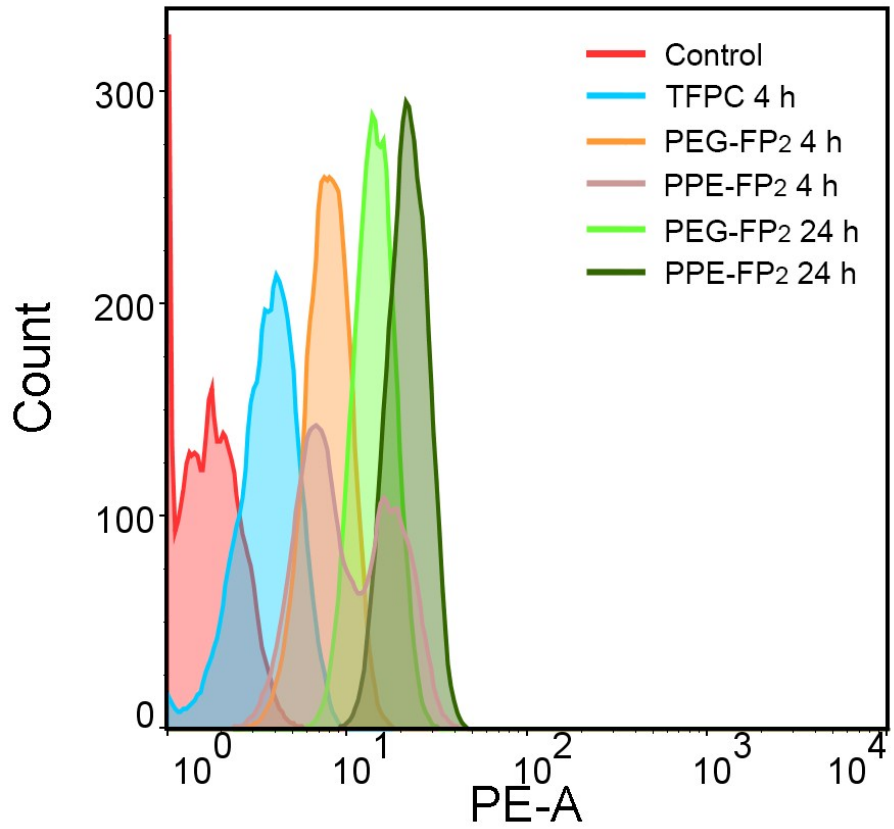


Fig. S20. Flow cytometry analyses of cellular internalization of **PPE-FP$_2$** and **PEG-FP$_2$** nanoparticles.

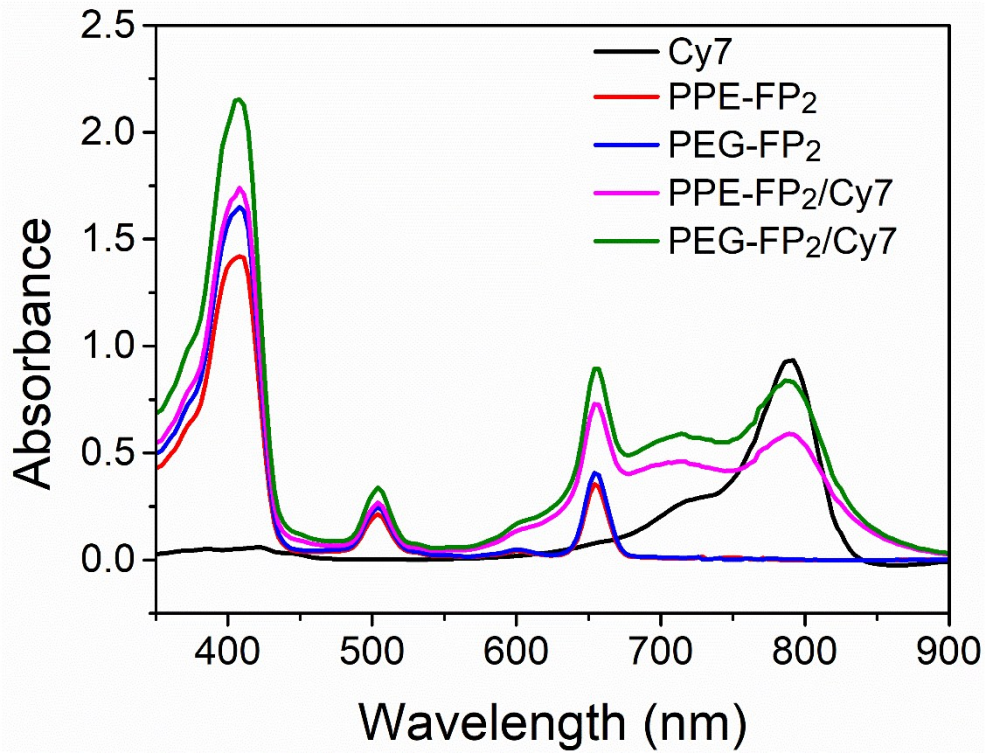


Fig. S21. UV spectra of nanoparticles labelled with or without Cy7.

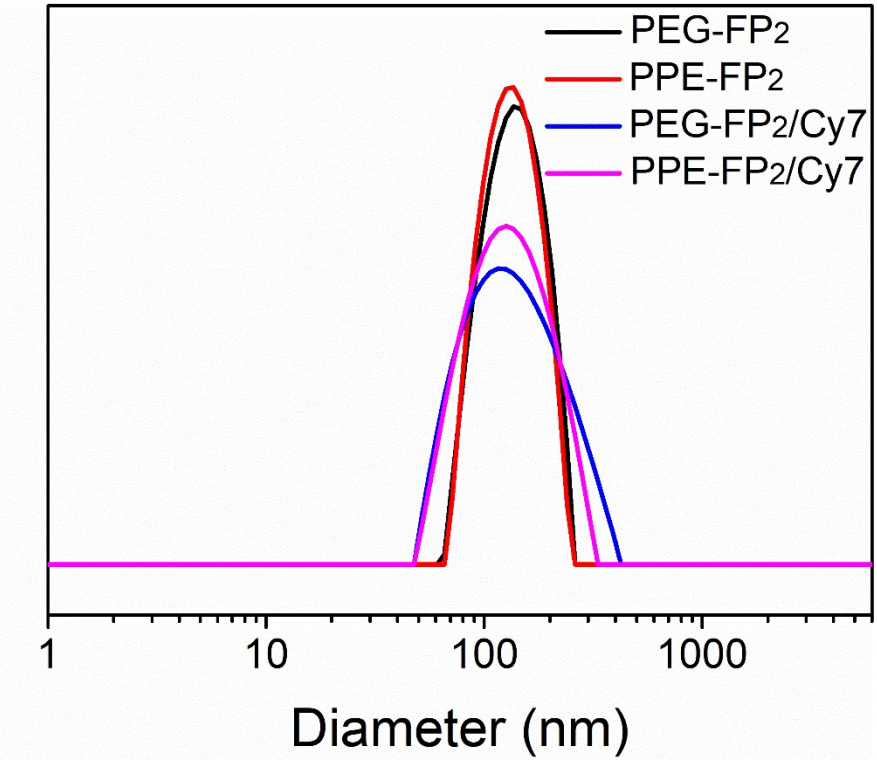


Fig. S22. Size distributions of Cy7-labeled nanoparticles determined by dynamic light scattering.

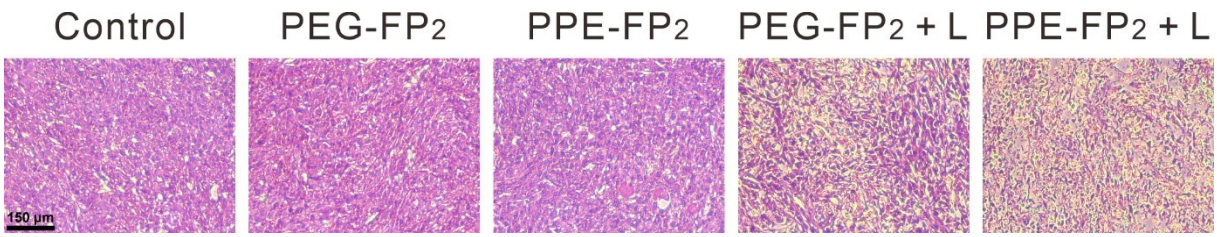


Fig. S23. Histological data (haematoxylin and eosin stained images) obtained from the isolated tumors.

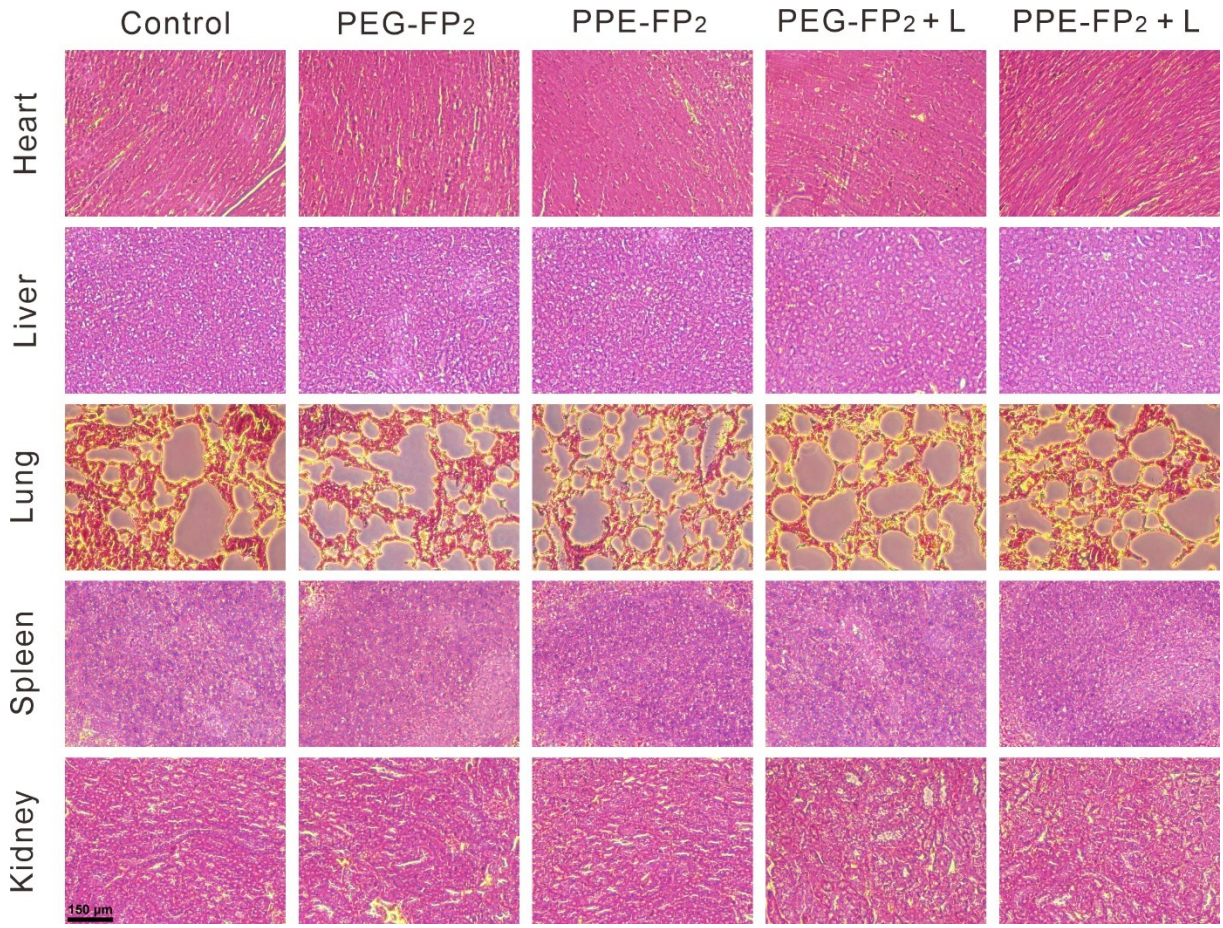


Fig. S24. Histological data (haematoxylin and eosin stained images) obtained from the liver, spleen, kidney, heart and lung.

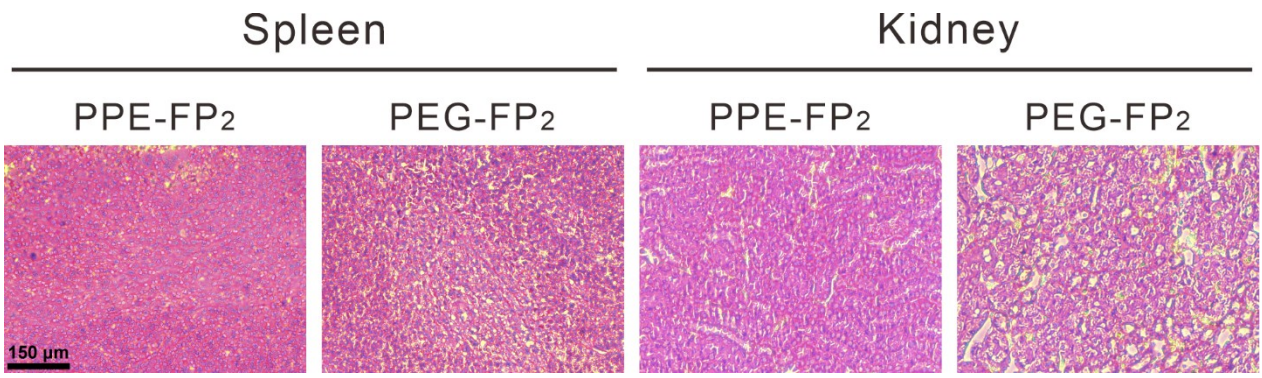


Fig. S25. Toxicity assessment of **PEG-FP$_2$** and **PPE-FP$_2$** nanoparticles *in vivo* after repeated administration at a high concentration.