Electronic Supplementary Information (ESI)

Anatase TiO_{2-x} and zwitterionic porphyrin polymer-based nanocomposite for enhanced cancer photodynamic therapy

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1. Experimental

1.1. Materials and instruments

All commercially sourced chemicals and solvents were used without further purification. Meso-5,10,15,20-tetra(4-hydroxylphenyl) porphyrin (THPP) was purchased from Frontier Scientifc, Inc. Fumaryl chloride (FA, purity: 95%), L-cysteine hydrochloride monohydrate (purity 99%), diethylene glycol (DEG, purity: 99%), hydrofluoric acid (HF, purity: 40% for analytical pro) and 1,3-diphenylisobenzofuran (DPBF, purity: 97%) were bought from J&K Scientifc Ltd. Triethylamine (TEA, purity: 99.5%) and methoxy polyethylene glycols, with the number average molecule weight of 2000 in PEG portion (CH_3-PEG2000-OH) were purchased from InnoChem. Pyridine and tetrabutyl titanate ($Ti(OBu)_4$), acetic acid, sodium acetate anhydrous and sodium chloride were bought from Beijing Chemical Works. Ethanol and ether were purchased from Concord Technology. Sodium dihydrogen phosphate dihydrate was brought from Sinopharm Chemical Reagent. Disodium hydrogen phosphate dodecahydrate was purchased from Xilong Scientific. Dialysis bags (MWCO: 1,000 Da and 3,500 Da) were bought from Viskase. Nitric acid (HNO_3, purity: 65-68%, BV-III) was purchased from Beijing Institute of Chemical Reagents. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were bought from Aladdin. 7404DDP cells (drug-resistant liver cancer cells) were obtained

from the cell bank of the Chinese Academy of Sciences (Shanghai, China).

^{1}H NMR spectra were measured using Bruker AVANCE AV400 NMR spectrometer. Fourier transform infrared spectroscopy (FT-IR) spectra were recorded on a Nicolet 6700 spectrometer. The type of muffle furnace is FO311C, Yamato Scientific Co., Ltd., Japan. UV-visible absorption spectra of THPP, TFP2000, TFPC or TiO_{2-x}@TFPC and DPBF mixed solution in ethanol were obtained using a UV-visible spectrophotometer (Shimadzu UV-2600), and UV-visible spectra of TiO_{2-x} was recorded on the UV-visible spectrophotometer (Shimadzu UV-2600) with integrating sphere accessories. X-ray photoelectron spectroscopy (XPS) measurements were carried out by using ESCALab250Xi multifunctional X-ray photoelectron spectrometer. X ray diffraction pattern was recorded using Empyrean HTK1200N polycrystalline X-ray diffractometer in the diffraction angle range 2θ=20-70°. The light source is a Cu/Kα radiation source (λ=0.154 nm), the voltage is 40 kV, and the current is 40 mA. The detector is a 1D line detector. The TiO_{2-x} particle morphology was examined by transmission electron microscope (TEM) using the JEM-1011 TEM operated at the accelerating voltage of 100 kV and the JEM 2100F TEM operated at the accelerating voltage of 200 kV. The TFPC nanoparticle and TiO_{2-x}@TFPC nanocomposite morphology were examined by TEM using the JEM-1011 TEM operated at the accelerating voltage of 100 kV. The samples were prepared by dropping 7 μL of

nanoparticles suspension on the copper grid followed by staining with 7 μL of phosphotungstic acid (20 mg mL^{-1}). Moreover, TiO_{2-x}@TFPC nanocomposite morphology were examined by a scanning electron microscope (SEM) using the JSM-6700F SEM operated at the accelerating voltage of 5.0 kV. The TiO_{2-x}@TFPC nanocomposites samples were prepared by solid powers or dropping nanocomposites aqueous solution followed by air-drying. Nanoparticles and nanocomposite size and polydispersity were determined using dynamic light scattering (DLS) at 25 ℃ by a Malvern Zetasizer Nano ZS90 equipped with a 633 nm vertically polarized He-Ne laser using back-scattering detection. The diffraction angle is 90°. Measurements were done in triplicate at 25 ℃. An inductively coupled plasma mass spectrometer (ICP-MS, Thermo iCAP RQ, Thermo Fisher, USA) was used for quantitative determination of trace levels of Ti concentration in the prepared TiO_{2-x}@TFPC nanocomposite aqueous solution and dialysis samples in drug release experiments. The thermodynamic properties of the TFPC and TiO_{2-x}@TFPC were measured by Thermo Gravimetric Analyzer (TGA 8000, N_2, PerkinElmer, USA). The irradiation source 650 nm laser is the high stability red diode laser (MRL-III-FS-650, Changchun New Industries Optoelectronics Technology Co., Ltd., China). The maximum output power of laser is 1 W. And the 630 nm laser irradiation was examined by using PDT630-B semiconductor laser photodynamic therapy machine (Laser Medical

Techonology Co., Ltd., China).

1.2. Synthesis of TFP2000

THPP-FA-PEG2000 (termed TFP2000) was synthesized via a one-pot esterification reaction, as shown in Scheme S1, ESI. And through adjusting the feed ratio, the reaction can obtain the TFP2000 with different molecular weights. In brief, THPP (0.11 mmol), FA (0.5 mmol) and TEA (2.5 mmol) were dissolved in 10 mL anhydrous dichloromethane (CH_2Cl_2), and then stirred at room temperature in dark. After 24 h reaction, CH_3-PEG2000-OH (0.6 mmol) was added into the above reaction mixture and remained at room temperature for another 24 h. Afterward, the reaction mixture was condensed by rotary evaporator and then dissolved in ethanol. Later, the residuals were subjected into the dialysis bag with the molecular weight cut-off (MWCO) of 3,500 Da against a mixed solvent of ethanol/Milli-Q water (volume ratio v/v, 3/1, 2/1, 1/1, 0/1, 0/1 and 0/1 in turn) for 48 h. Finally, the obtained product of TFP2000 was lyophilized. The final lyophilized product was the brown fluffy solid with a yield of 21.94% and stored in a desiccator. ^{1}H NMR, δ (400 MHz, DMSO-d_6, TMS, ppm): the protons of the THPP porphyrin portion: 8.95, 8.38, 8.31, 8.09, 7.77, 7.24, 7.14.

1.3. Synthesis of TFPC

The detailed process for the synthesis of zwitterionic covalent organic polymer TFPC is described as follows. 21.7 mg of polyester TFP2000 (containing 0.062 mmol of C=C theoretically, 1 eq.) and 54.4 mg of L-cysteine hydrochloride monohydrate (containing 0.31 mmol thiols, 5 eq.) were dissolved in 3 mL DMF. The solution was degassed for 10 min, and then refilled with N_2. 25 μL of pyridine (5 eq.) was added into the solution as the catalyst. The reaction mixture was stirred for 12 h at room temperature in dark. The reaction solution was then placed in a dialysis bag (MWCO: 1,000 Da). The dialysis bag was put into water for dialysis for 2 days in order to remove the excess functional thiols, catalyst and solvent. The dialysis solution was finally lyophilized to obtain the light green fluffy solid TFPC. This product was stored in a desiccator.

1.4. Synthesis of TiO_{2-x}

The anatase TiO_{2-x} was synthesized according to an approach from the literature.[25] 0.1 mol $Ti(OBu)_4$ (3.4 g) was mixed with 25 ml DEG followed by 30 minutes stirring at 1,200 rpm leading to the formation of titanium glycolate gel. 0.4 mol of water (7.2 ml) was added to facilitate sufficient hydroxylation, and the obtained solution was stirred continued for further 15 minutes. The hydrated titanium glycolate gel that has been formed was kept in a muffle furnace at 300 °C for 2 hours. The solid crystals obtained after rapid cooling to room temperature was washed using 50 ml each of

ethanol, ether and water to remove the organic and inorganic impurities. Finally, the obtained solid was dried for 12 h at 50 °C.

1.5. Detection of THPP content in TFPC

The THPP content in covalent organic polymer TFPC was determined according to approach of the literatures.[14,15] In general, the lyophilized powders of TFPC and THPP were dissolved in ethanol to determine the detected wavelength through UV-visible spectrum scanning. The detected wavelength is 419 nm. A series of standard ethanol solutions of THPP (0.125-1.5 μg mL^{-1}) were prepared. Then a standard curve was drawn using these solutions with different THPP concentrations through spectrometry determination at the detected wavelength (Fig. 1B). And the change of the ultraviolet absorption of the polymer TFPC is caused by the containing of THPP with porphyrin ring. Finally, the THPP contents were calculated through the UV absorption of TFPC ethanol solution. The THPP contents (TC%) was calculated using the following formulae:

$$TC\% = A/B \times 100\%$$

It should be noted that A is the concentration calculated from the standard curve with the detected UV absorption value of TFPC ethanol solution, B is the concentration of TFPC ethanol solution.

1.6. Characterization of the synthesized TiO_{2-x}

The TiO_{2-x} samples were characterized using XPS, XRD, UV-visible spectroscopy and TEM.

1.7. Preparation of nanoparticles TFP2000 and TFPC, and nanocomposite TiO_{2-x}@TFPC

To prepare the nanoparticles of TFP2000, 0.9 mg of lyophilized covalent organic polymer TFP2000 was dissolved in ethanol (590 μL). The ethanol was added to water in a sample bottle and the solution was stirred. The volume ratio of ethanol to water is 1/3. Finally, TFP2000 solution was purified by dialysis with a dialysis bag (MWCO: 1,000 Da) in water for 12 h to get the 0.4 mg mL^{-1} nanoparticle aqueous solution. The preparation of 0.4 mg mL^{-1} TFPC nanoparticles aqueous solution was the same as that of TFP2000.

To obtain the nanocomposite TiO_{2-x}@TFPC, the TiO_{2-x} nanoparticles (5 mg) was mixed with 5 mL of ethanol solution containing 20 mg of TFPC in a sample bottle. The mixture was stirred for 12 h at room temperature in dark and then purged with continuous dry nitrogen gas for 12 h to slowly evaporate the ethanol. Subsequently, 5 mL of deionized water was added into the mixed powders and sonicated for 10 min to obtain the crude product nanocomposite TiO_{2-x}@TFPC. The dispersion was then purified by dialysis with a dialysis bag (MWCO: 1,000 Da) in water for 12 h. Finally, the obtained nanocomposite aqueous solution was centrifuged at

low speeds (4,000 rpm, 3 min) and high speed (8,000 rpm, 3min and 16,000 rpm, 3 min in turn) to purify for removing the possible formation of large aggregates.

The preparation of nanoparticles TFPC and nanocomposite TiO_{2-x}@TFPC in the physiological environment (PBS buffer solutions at pH 7.4) followed a similar procedure except the solution was PBS buffer solution (pH = 7.4).

1.8. Characterization of nanoparticles TFP200 and TFPC, and nanocomposite TiO_{2-x}@TFPC

The samples TFP200 and TFPC, and TiO_{2-x}@TFPC were characterized using DLS, TEM or SEM.

1.9. Thermal stability of TFPC and TiO_{2-x}@TFPC

The thermal stability of the materials was characterized by thermal gravimetric analysis. The sample was heated from 50 °C to 750 °C at a heating rate of 20 °C min^{-1} under a nitrogen atmosphere. The curves of weight loss versus temperature were recorded.

1.10. Detection of Ti concentration of the TiO_{2-x}@TFPC nanocomposite aqueous solution

A 10 μL of the prepared TiO_{2-x}@TFPC nanocomposite solution was

digested with 3 mL of HNO_3 and HF mixed aqueous solution (the volume ratio, HNO_3: HF: H_2O = 2: 0.2: 100) for 4 h, the obtained liquid was diluted with 2 mL HNO_3 and HF mixed aqueous solution, and then detected by ICP-MS to obtain the Ti concentration.

1.11. Drug release from TiO_{2-x}@TFPC nanocomposites

The *in vitro* release experiment process of TiO_{2-x}@TFPC nanocomposites is described as follows. 1 mL of the nanocomposite solution was transferred to a dialysis bag (MWCO: 3500 Da) and 30 mL of the release medium solution was added to a bottle. The dialysis bag containing the nanocomposite solution was then placed into the bottle. The bottle was then placed in a shaker with a speed of 110 rpm at 37 °C to shake for releasing the TiO_2 in the dark. Three solutions selected in this experiment were PBS buffer solution (pH = 7.4), acetic acid buffer solutions (pH = 6.5 and pH = 5.5). The release medium solution was extracted at each predetermined time interval and 3 mL of corresponding fresh buffer solution was then added to keep the total volume constant in each release system. The Ti contents with different release conditions were determined by ICP-MS and the Ti content at each time point was recorded as A_t. In addition, 1 mL of the nanocomposite solution with different release conditions was diluted to 30 mL and shaken for 48 h in a shaker. 3 mL of the solution was then used to determine the Ti content by ICP-MS, which was recorded as A_0.

Therefore, the cumulative amount of Ti released (expressed as DR%) at each time point is calculated using the following formula:

$$DR\% = A_t/A_0 \times 100\%$$

1.12. Detection of singlet oxygen generation capacity of nanoparticle TFPC and nanocomposite TiO_{2-x}@TFPC

To study the photodynamic effects of nanoparticle TFPC and nanocomposite TiO_{2-x}@TFPC, TFPC and TiO_{2-x}@TFPC were mixed with a singlet oxygen trap DPBF (the mass ratio of TFPC or TiO_{2-x}@TFPC with DPBF was 1.16:1), then irradiated with the 650 nm laser. And the samples were irradiated with the light source from a 4 cm distance. All samples were homogenized for 1 min before measurements. The ROS from TFPC or TiO_{2-x}@TFPC was measured by the reaction between TFPC or TiO_{2-x}@TFPC solution and DPBF solution in ethanol and examined by monitoring the decrease of the absorption at 419 nm. The mixture was kept at 37 °C during the laser irradiation. Furthermore, under the 630 nm laser irradiation (50 mW cm^{-2}) with different periods of irradiation time (15 and 30 min), absorbance of the DPBF of TFPC ethanol solutions was also recorded. To compare the photodynamic effects of nanoparticle TFPC and nanocomposite TiO_{2-x}@TFPC, pure ethanol was recorded as control. The absorption spectra were recorded and repeated independently three times.

1.13. In vitro cytotoxicity assay of the nanoparticle TFPC and nanocomposite TiO_{2-x}@TFPC

7404DDP cells were grown in DMEM (HyClone) supplemented with 10% fetal bovine serum, 0.03% L-glutamine and 1% penicillin/streptomycin in 5% CO_2 at 37 °C.

The *in vitro* cytotoxicity assays of the nanoparticle TFPC and nanocomposite TiO_{2-x}@TFPC were evaluated by the methyl thiazolyl tetrazolium (MTT) assay. 7404DDP cells harvested in a logarithmic growth phase were seeded in 96-well plates at a density of 4×10^3 cells per well and incubated in DMEM overnight. 10 μL of the TFPC nanoparticle and TiO_{2-x}@TFPC nanocomposite aqueous solutions (at a final concentration ranging from 2.3 to 36.4 μg mL^{-1}) was added. The incubation time for the TFPC nanoparticle and TiO_{2-x}@TFPC nanocomposite was 24 h. The 7404DDP cells were irradiated with the laser (650 nm, 300 mW) light source from a 4 cm distance for 15 min. After incubation for an additional 12 h, 10 μL of MTT solution (the concentration is 5 mg mL^{-1} in PBS) was added to every well and the plates were incubated for another 4 h at 37 °C, followed by addition of 100 μL of sodium dodecyl sulfate solution (10 wt% in PBS) to each well to dissolve the formazan crystals. Finally, the plates were shaken for 12 h in dark, and the absorbance of formazan product was measured at 570-650 nm by a microplate reader. The MTT assay of the TFPC nanoparticles and TiO_{2-x}@TFPC

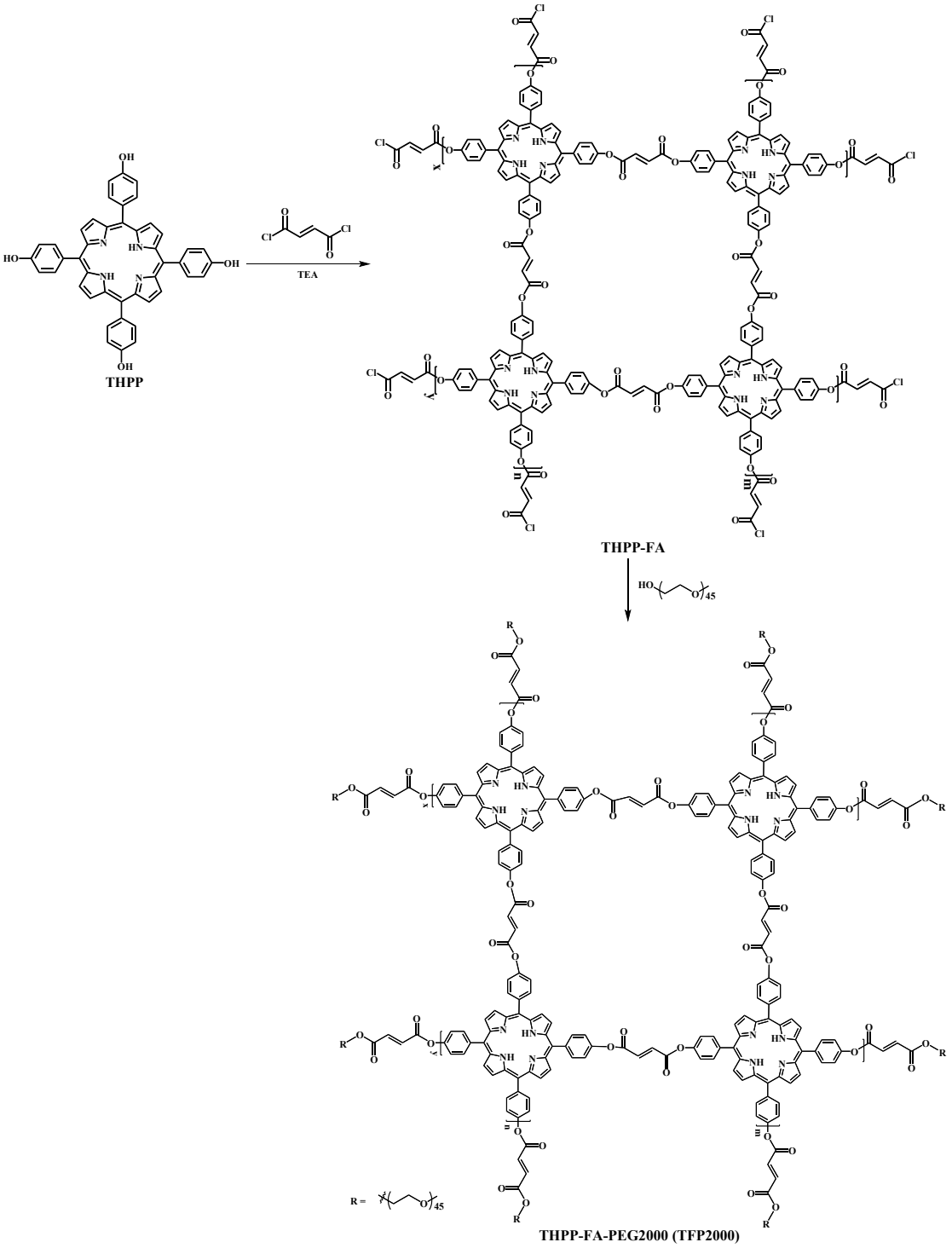
nanocomposite to 7404DDP cells for evaluating the cytocompatibility of these nano-systems followed a similar procedure except the added sample solutions at the final concentrations ranges from 45.5 μg mL^{-1} to 272.7 μg mL^{-1} and no laser irradiation.

The relative cell viability (%) of 7404DDP was all determined by comparing each absorbance at 570 and 650 nm. Data are presented as average ± SD (n = 6).

1.14. Statistical analysis

The results are presented as mean ± standard deviation of three separate experiments. Two-tailed Student's t-test, and groups were determined as significantly different with p values less than 0.05.

2. Supplementary figures



Scheme S1 Synthesis of the porphyrin based covalent organic polymer.

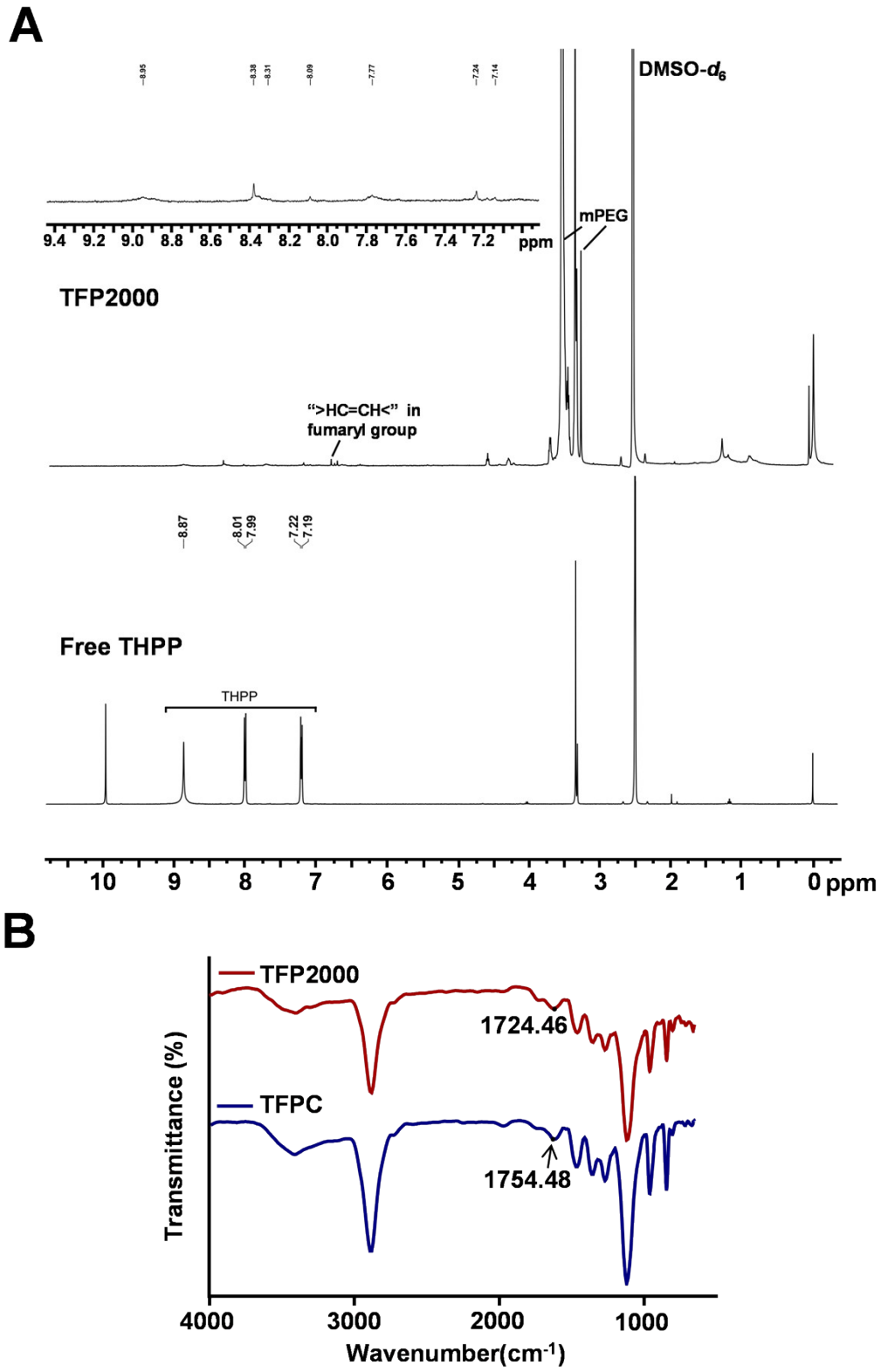


Figure S1 (A) ^{1}H NMR spectra of TFP2000 (above) and Free THPP (below) in DMSO-d_6. (B) FT-IR of the TFP2000 and TFPC.

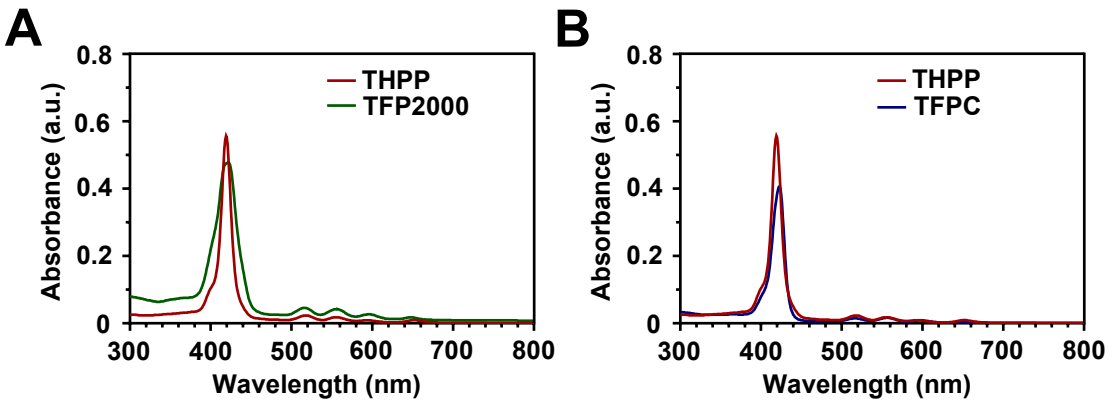


Figure S2 UV-vis spectra of (A) free THPP (1.0 μg mL^{-1}) with TFP2000 (200.0 μg mL^{-1}), and (B) free THPP (1.0 μg mL^{-1}) with TFPC (50.0 μg mL^{-1}).

Table S1 Detection results of THPP content in TFPC by UV-vis spectra.

Sample	Concentration in ethanol (B, μg/mL)	Absorbance in 419 nm	THPP porphyrin portion calculated concentration (A, μg/mL)	THPP porphyrin portion contents (wt%)
TFPC	**50.0**	**0.369**	**0.895**	**1.79%**

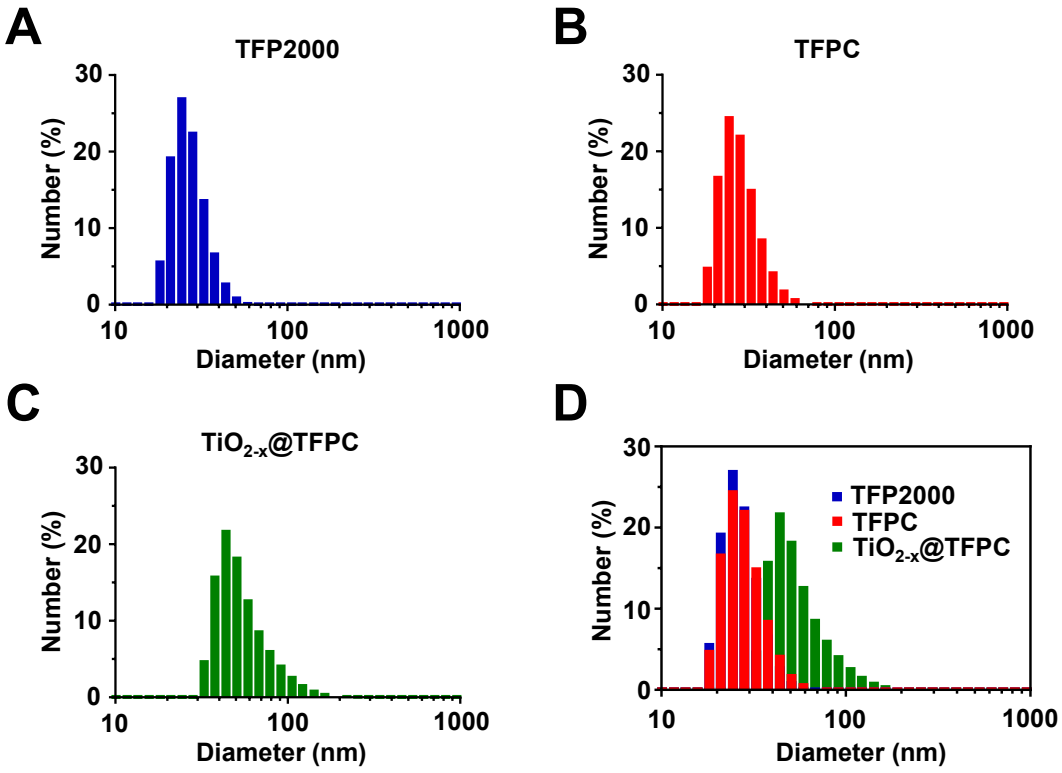


Figure S3 Hydrodynamic diameter of (A) TFP2000 nanoparticles, (B) TFPC nanoparticles and (C) TiO_{2-x}@TFPC nanocomposites in aqueous solution. (D) is the hydrodynamic diameter superposition diagram.

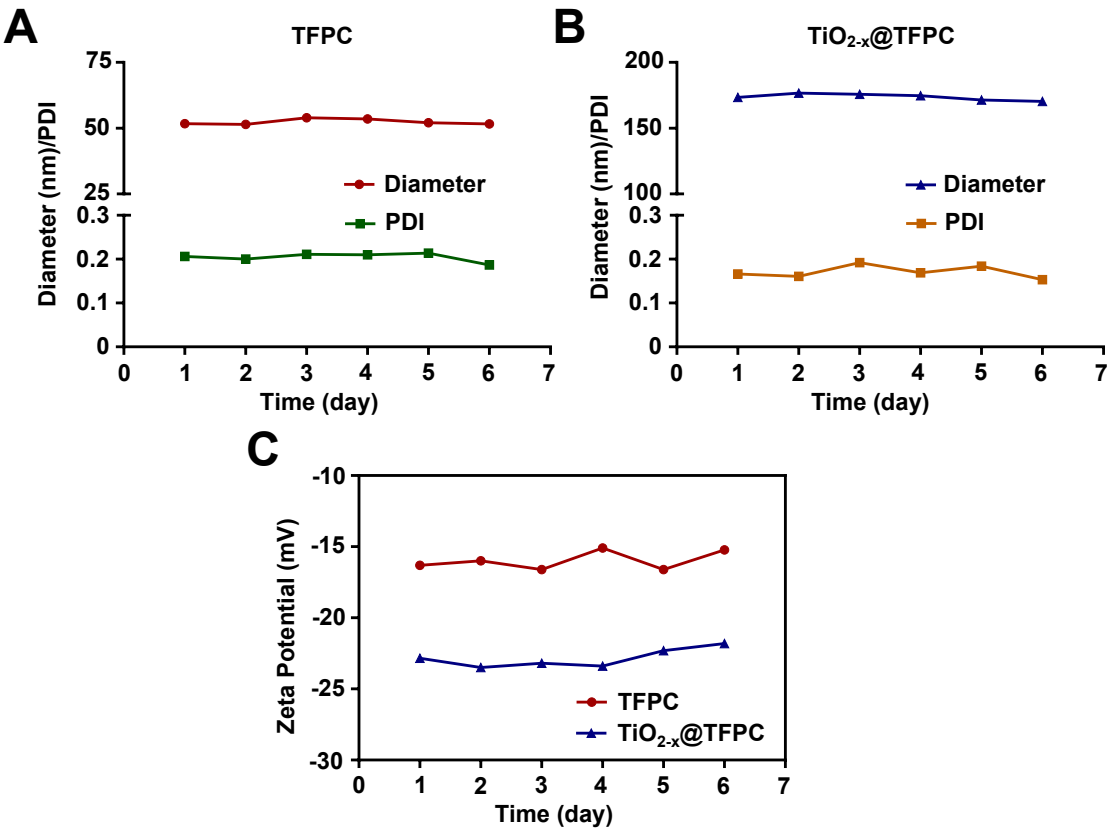


Figure S4 The stability and zeta potential of the nanoparticles in the physiological environment (PBS buffer solutions at pH 7.4). Hydrodynamic diameters change of (A) TFPC nanoparticles and (B) TiO_{2-x}@TFPC nanocomposites for 6 days. (C) ζ-potential values change of TFPC or TiO_{2-x}@TFPC for 6 days.

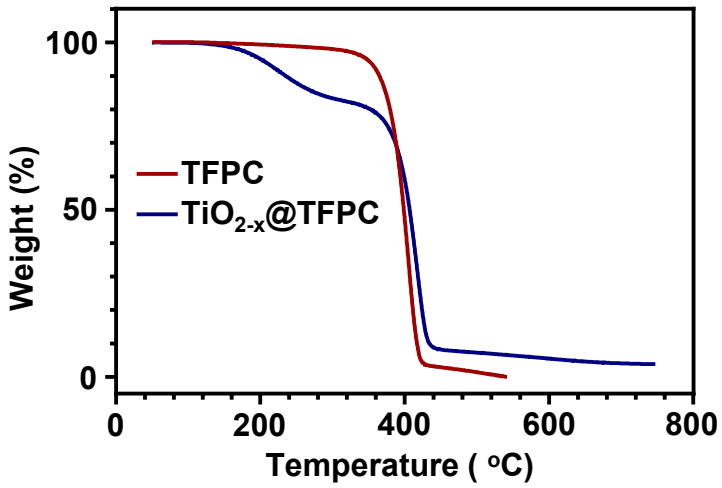


Figure S5 The thermostable curves of the lyophilized TFPC and TiO_{2-x}@TFPC nanoparticles analyzed by thermogravimetric analyzer.

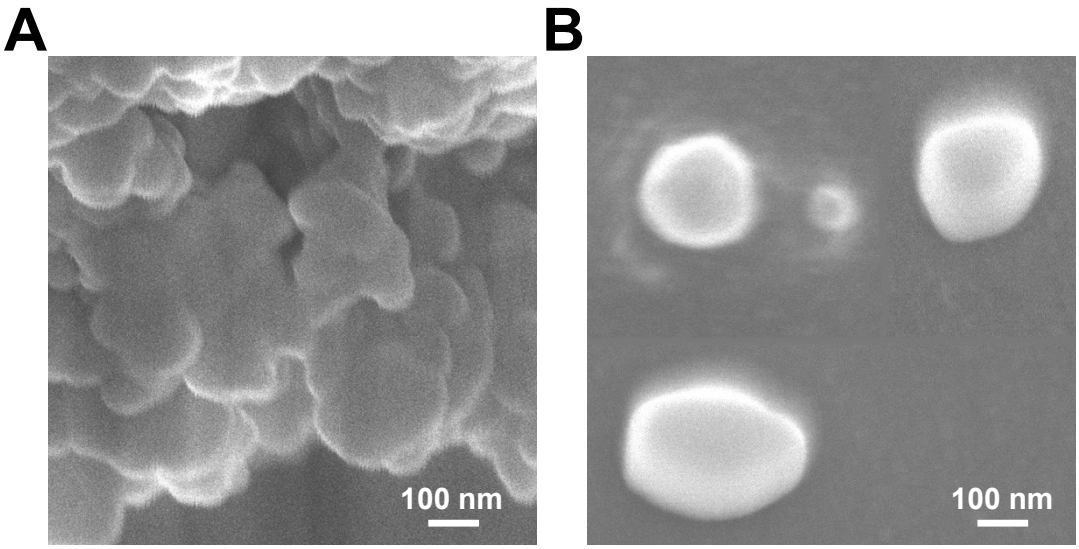


Figure S6 SEM images of (A) the lyophilized TiO_{2-x}@TFPC powders and (B) TiO_{2-x}@TFPC nanocomposites prepared by aqueous solution.

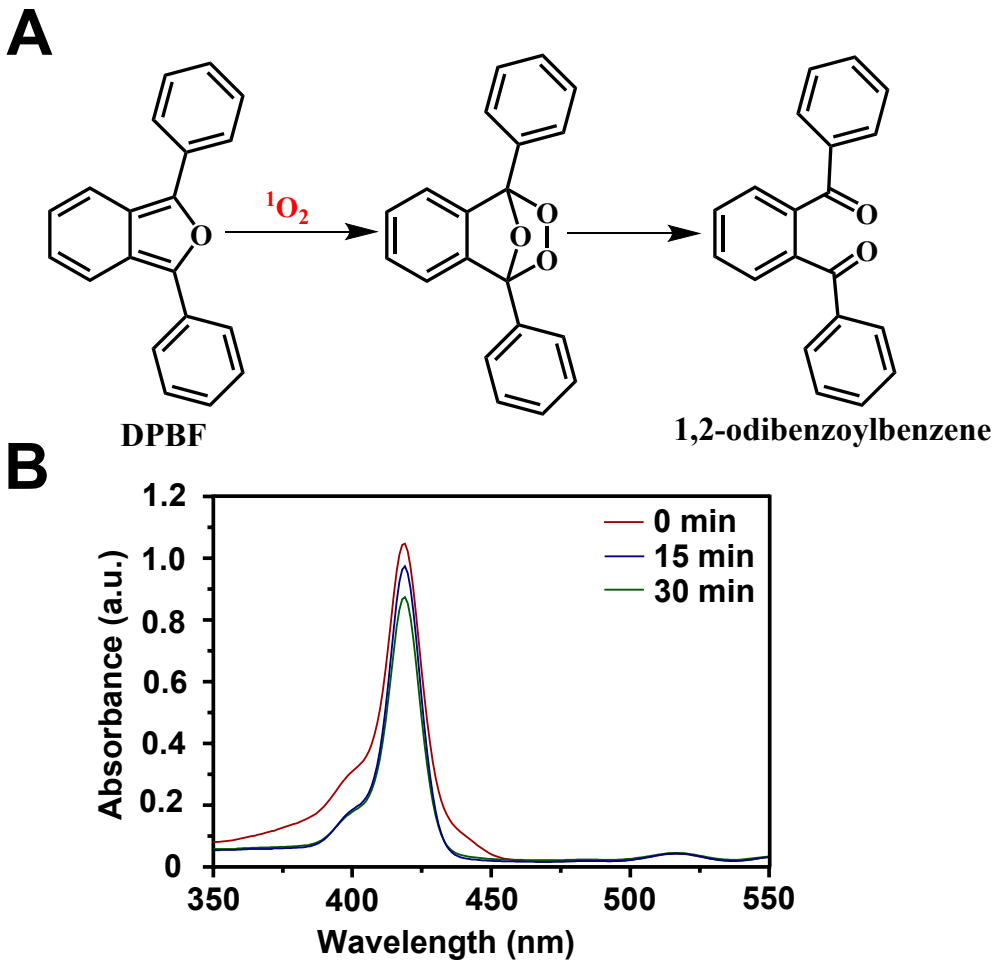


Figure S7 (A) The chemical reaction equation of singlet oxygen and capture agent DPBF. (B) Photodecomposition of DPBF by $^1O_2$ under irradiation by a 630 nm laser at 50 mW cm^{-2}, in presence of TFPC ethanol solution (25 μg mL^{-1}). Time-dependent decrease in the absorbance of DPBF at 419 nm with the mixed ethanol solution of TFPC.

Table S2 The photodynamic performance of the TiO_{2-x}@TPFC and other respective nano-photosensitizers.

	Structure [a]	Laser [nm]	Laser dose	PSs dose [μg mL^{-1}]	Irradiation time [min]	η [%] [b]	Ref.
TFP-NN	**Porphyrin-based, NP**	**638**	**1 W cm^{-2}**	**7.13**	**60**	**8.3**	**1**
NYF@Ti	**$NaYF_4$:Yb^{3+},Tm^{3+}, TiO_2, UCNP**	**980**	**- [c]**	**320**	**8**	**49**	**2**
UMOF-TiO_2	**Porphyrin-based, TiO_2, UCNP**	**980**	**0.72 W cm^{-2}**	**1000**	**30**	**34.1**	**3**
TSPP-TiO_2	**P25, tetra sulphonatophenyl porphyrin, NC**	**500~ 550**	**-**	**-**	**15**	**62.0**	**4**
[Sn(TPrPc)Br_2]	**[Sn(TPrPc)Br_2] complex**	**> 600**	**200 W**	**4.53**	**83**	**≈ 82**	**5**
DVDMS	**Sinoporphyrin sodium, porphyrin dimer**	**635**	**5 J**	**4924.96**	**-**	**39.1**	**6**
TiO_{2-x}@TFPC	**TiO_{2-x}, porphyrin-based, NC**	**650**	**300 mW**	**6.98**	**30**	**82.6**	**This work**

[a] Abbreviations: NP, nanoparticle; UCNP, upconversion nanoparticle; NC, nanocomposite.

[b] η is expressed as the rate of DPBF decomposition.

[c] (-) is expressed as that this data is not specified in reference.

Scheme S2 The following equations are about the light activated porphyrin (PD) groups in TFPC generating singlet oxygen ($^1O_2$). The processes may take place:[7]

$$PD + h\nu \rightarrow {}^1PD^* \qquad (1)$$

$$^1PD^* \rightarrow {}^3PD^* \qquad (2)$$

$$^3PD^* + {}^3O_2 \rightarrow {}^1O_2 + PD \qquad (3)$$

Here (*) is expressed as in the type of excited state.

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