Oxidative burst as a continuous H_2O_2 self-supplier for tumor oxygenation in photodynamic therapy

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Chemicals: Dulbecco's minimum essential medium (DMEM) was from Gibico. New bovine serum (NBS) was Sijiqing. BES-H_2O_2-AC probe was from Wako pure chemical industries. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was from Amrosco. Singlet oxygen sensor green (SOSG), Lyso-tracker blue was from Thermofisher. Tris (4,7-diphenyl-1,10-phenanthroline) ruthenium (II) dichloride [$Ru(dpp)_3Cl_2$] was from Alfa Aesar. Annexin V-FITC/PI kit, OCT embedding medium, pimonidazole hypoxyprobe-1, CD 31 antibody, horseradish peroxidase-connected anti-rabbit IgG, anti-mouse IgG1, and haematoxylin and eosin (H&E) kit were from Yeasen Biotechnology Co. Ltd. ZnPc1 was from Tokyo Chemical Industry Co. Ltd. $FeCl_3 \cdot H_2O$, sodium acetate, and histidine were from Sinopharm Group Co. Ltd. Methoxypoly(ethylene glycol) acrylate (PEG) was from ShangHai ToYong.

Characterization: Ultraviolet-Visible (UV-Vis) absorption spectra were obtained using a Varian Cary 50 spectrophotometer (USA). Fluorescence spectrophotometer was measured by Varian Cary Eclipse (USA). Zeta potential and dynamic light scattering (DLS) was obtained

using Malvern Zetasizer Nano 90 (UK). The transmission electron microscopy (TEM) images were obtained from a HITACHI H7650 transmission electron microscope (80 KV) (Japan). In vitro fluorescence images were detected using a fluorescence inversion microscope system and confocal laser scanning microscopy (CLSM, Nikon, Japan). Relaxivity and nuclear magnetic resonance imaging (MRI) was obtained by NIUMAG MesoMR23-60H-I magnetic resonance imaging system. In vivo fluorescence images were obtained using Series III IVIS Lumina K. Cell apoptosis was detected using a flow cytometer (FCM, Beckman, USA). Portable dissolved oxygen tester (JPBJ-608, Shanghai, China).

Synthesis of ZnPcs: ZnPc3, ZnPc4, ZnPc8, ZnPc9, ZnPc10, ZnPc11 and ZnPc12 was synthesized as previous reports.[1-6] The synthesis methods of ZnPc2, ZnPc5, ZnPc6 and ZnPc7 were listed as follows and synthesis routes were shown as follows.

Synthesis of ZnPc2: 4- nitrophthalonitrile (1.50 g, 8.66 mmol), p-hydroxybenzaldehyde (1.59 g, 13.00 mmol) and K_2CO_3 (2.39 g, 17.33 mmol) were added DMF (10 mL) under N_2 protection. The compound was stirred and heated 70 °C for 4.5 h. When it was cooled down to the room temperature, the reaction solution was poured into NaOH solution and the light yellow solid precipitated out. Then the solid was filtered and washed to achieve the yellow product (A1). A1 (1.00 g, 4.03 mmol) was dissolved in ethanol (30 mL) and sodium borohydride (167.65 mg, 4.43 mmol) was added above solution to react for 2 h under stirring. The product was filtered, dried and subjected to column chromatography with ethyl acetate: petroleum ether = 1:1 (v:v) as a developing solvent. Then the oily brick-red sample was afforded (A2). Under N_2 protection, A2 (1.00 g, 4 mmol) and zinc acetate (458 mg, 2.50 mmol) was added in n-pentanol (6 mL). Then the mixture was stirred and raised the temperature to 100 °C. After fully dissolution, DBU (300 μL) was added into the reactant and the temperature was heated to 130 °C for 12 h. The n-pentanol was removed by decompress distillation and the dark green crude sample was dissolved in the mixture of methanol and dichloromethane. Ethyl acetate (EA): petroleum ether (PE) = 2:1 and methanol: dichloromethane = 1:3 (v:v) were used for gradient elution and the finally pure dark green solid was dried by vacuum oven (ZnPc2). M. P. > 300 °C. IR (KBr, cm^{-1}): 3415 , 2928, 1600, 1473, 1391, 1340, 1228, 1047, 824, 747.^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm): 8.88 (s, 6H, Pc-H), 8.44 (d, 6H, J=7.6 Hz, Pc-H), 7.71-7.42 (m,

16H, Ar), 5.34 (d, 4H, J=4.4 Hz, OH), 4.65(s, 8H, CH2). HRMS (MALDI-TOF) m/z: 1066.90, (Calculated. For $C_{60}H_{40}N_8O_8Zn$: 1066.39). Anal. Calcd. For $C_{60}H_40N_8O_8Zn$: C, 67.58; H, 3.78; N, 10.51. Found: C, 67.53; H, 3.81; N, 10.48.

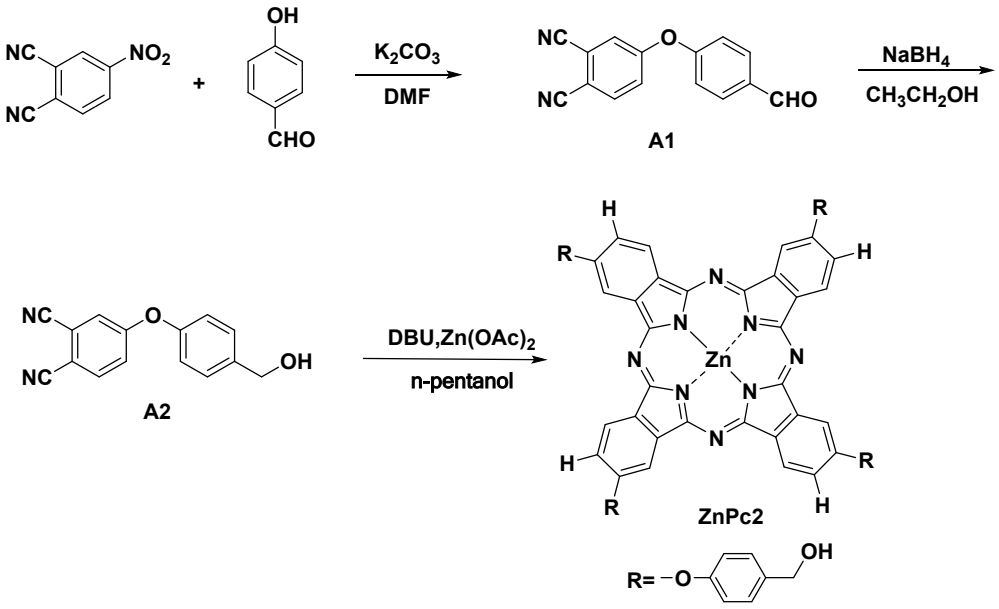


Fig. S1 The synthesis route of ZnPc2.

Synthesis of ZnPc5: P-nitrophenol (0.20 g, 1.16 mmol), 4-nitrophthalonitrile (0.48 g, 3.47 mmol) and K_2CO_3 (1.28 g, 9.24 mmol) were sequentially added to DMSO (10 mL) under the protection of N_2. The mixture was stirred and heated to 80 °C for 5 h. After cooling to the room temperature, the product was extracted by dichloromethane. The extraction layer was mixed with crude silica gel and purified by column chromatography with EA: PE = 1: 2 (v: v) as a developing solvent to obtain 4-(4-nitrophenoxy)phthalonitrile (B1). B1 (0.25 g, 0.94 mmol) and zinc acetate (0.01 g, 0.58 mmol) were added n-pentanol (8 mL) under the protection of N_2. The mixture was stirred and heated to 90 °C slowly. Then DBU (200 μL) was added to the above solution for reacting 30 min and heated to 140 °C under stirring condition for 24 h. After cooling down the room temperature, methanol (50 mL) was added for filtering. The filter cake was dissolved in dichloromethane and centrifuged. The precipitate was discarded and the crude product was obtained by rotary evaporation. Then the product was dissolved in methanol, centrifuged and dried in vacuum oven to afford a blue solid product ZnPc5 (0.12 g, 45.21%, calculated as A1). M. P. > 200 °C. IR (KBr, cm^{-1}): 3456, 1591, 1519, 1488, 1340, 1236, 1083,

947, 744. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm): 8.79 (s, 4H, Ar-H), 8.35 (br, 12H, J = 40 Hz, Pc-H), 7.87 (s, 4H, Ar-H), 7.56 (s, 8H, Ar-H). HRMS (MALDI-TOF): Calcd. For $C_{56}H_{28}N_{12}O_{12}Zn$: m/z=1126.27. Found: 1127.52. Anal. Calcd. For $C_{56}H_{28}N_{12}O_{12}Zn$: C, 59.72; H, 2.51; N, 14.92. Found: C, 60.01; H, 2.38; N, 14.79.

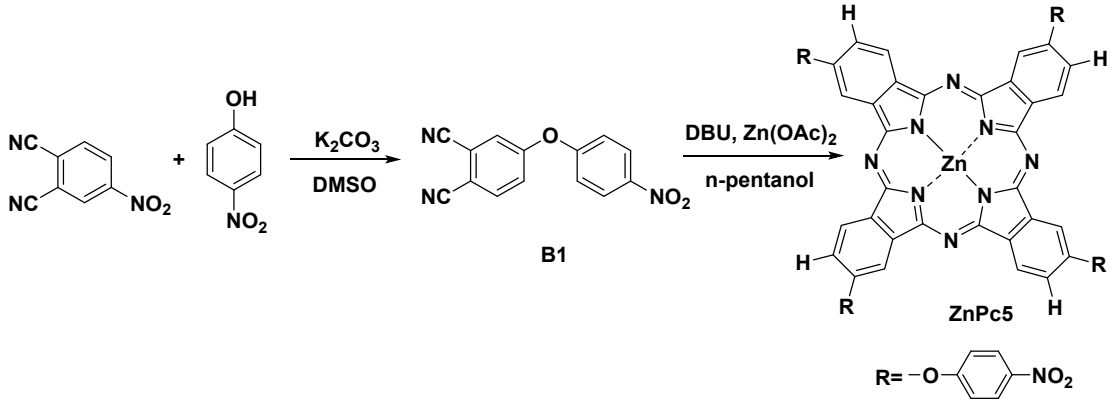


Fig. S2 The synthesis route of ZnPc5.

Synthesis of ZnPc6: P-hydroxybenzenesulfonic acid (0.36 g, 1.86 mmol), 4-nitrophthalonitrile (0.25 g, 1.44 mmol) and K_2CO_3 (0.30 g, 2.17 mmol) were added to DMF (30 mL) under N_2 protection. The mixture was stirred and heated to 45 °C for reacting 8 h. After cooling down, the mixture was filtrated and acetone (60 mL) was added. Then the precipitated white solid was collected by washing with absolute ethanol and distilled water and rotary evaporation. The crude product was recrystallized by methanol and dried in vacuum to obtain the pale yellow product 4-(4-sulfonic acid phenoxy)phthalonitrile (C1). C1 (0.25 g, 0.83 mmol) and zinc acetate (0.04 g, 0.22 mmol) were added in n-pentanol (10 mL) under N_2 protection and kept stirring until the temperature raised to 120 °C. Then DBU (300 μL) was added in the above solution for reacting 30 min and continued heating to 140 °C. After reacting 20 h, acetone (20 mL) was added and the solution was filtered. The dark green solid product was collected and washed several times with absolute ethanol. Then it was dried by vacuum oven and recrystallized by methanol to acquire the blue-green powder product ZnPc5 (0.11 g, 41.73%, calculated as B1). M.P. > 200 °C. IR (KBr, cm^{-1}): 3434, 1630, 1450, 1128, 1115, 1033, 700. ^{1}H NMR (400 MHz, D_2O): δ (ppm) 7.84 (s, 4H, Pc-H), 7.23 (t, 12H, *J* = 40.0 Hz, Ar-H), 6.90 (t, 8H, *J* = 14 Hz, Ar-H), 6.83 (t, 8H, *J* = 12 Hz, Ar-H). Anal. Calcd. For $C_{56}H_{32}N_8O_{16}S_4Zn$:

C, 53.11; H, 2.55; N, 8.85. Found: C, 52.98; H, 2.73; N, 8.64.

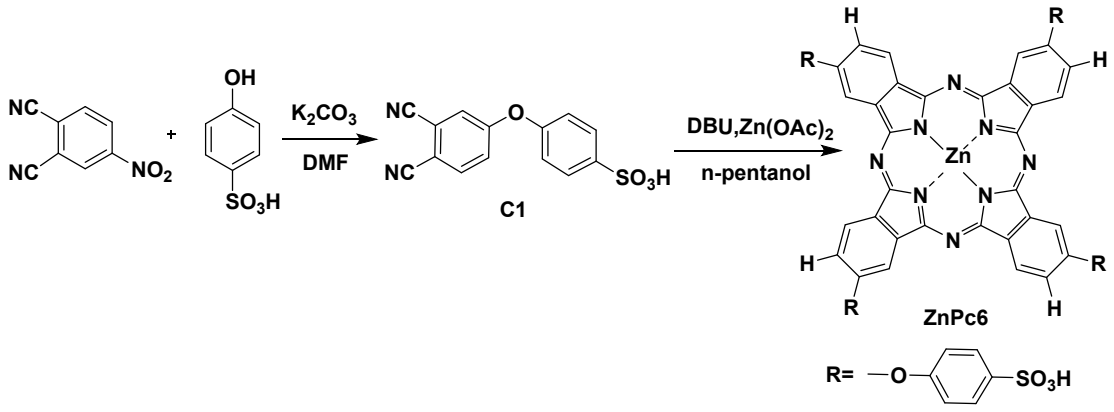


Fig. S3 The synthesis route of ZnPc6.

Synthesis of ZnPc7: P-tert-butylphenol (0.67 g, 4.48 mmol), 4-nitrophthalonitrile (0.48 g, 2.80 mmol) and K_2CO_3 (0.78 g, 5.60 mmol) were added in DMF (10 mL) and stirred at the room temperature for 6 h. Then distilled water (30 mL) was added and extracted by dichloromethane and saturated brine for three times each. The extract layer was dried with anhydrous sodium sulfate and the white powdery solid 4-(4-tert-butylphenoxy)phthalonitrile (D1) was obtained by rotary evaporated. D1 (0.87 g, 2.85 mmol) and zinc acetate (0.54 g, 2.99 mmol) were added n-pentanol (25 mL) under the protection of N_2. The mixture was slowly heated to 90 °C under stirring condition. Then DBU (1.5 mL) was added for reacting 30 min and the temperature was rapidly raised to 140 °C for stirring 24 h. After cooling to 60 °C, the above solution was filtered and the filter cake was washed with methanol. The filtrate was collected and extracted with methanol and acetone. Then the extraction layer was achieved by rotary evaporated and dried in vacuum oven to obtain a blue solid product ZnPc7 (0.28 g, 34%, calculated as C1). M.P. > 200 ℃. IR (KBr, cm^{-1}): 3050, 2960, 1600, 1360, 1230, 721. ^1H NMR (400 MHz, D6-DMSO): δ (ppm) 7.85 (s, 4H, Pc-H), 7.71-7.33 (m, 16H, Ar-H), 7.29-7.05 (m, 8H, Pc-H), 1.40-1.05 (m, 36H, CH_3). HRMS (MALDI-TOF): Calcd. For $C_{72}H_{64}N_8O_4Zn$: m/z=1170.71. Found: 1169.97. Anal. Calcd. For $C_{72}H_{64}N_8O_4Zn$: C, 73.87; H, 5.51; N, 9.57。 Found: C, 73.55; H, 5.78; N, 9.98.

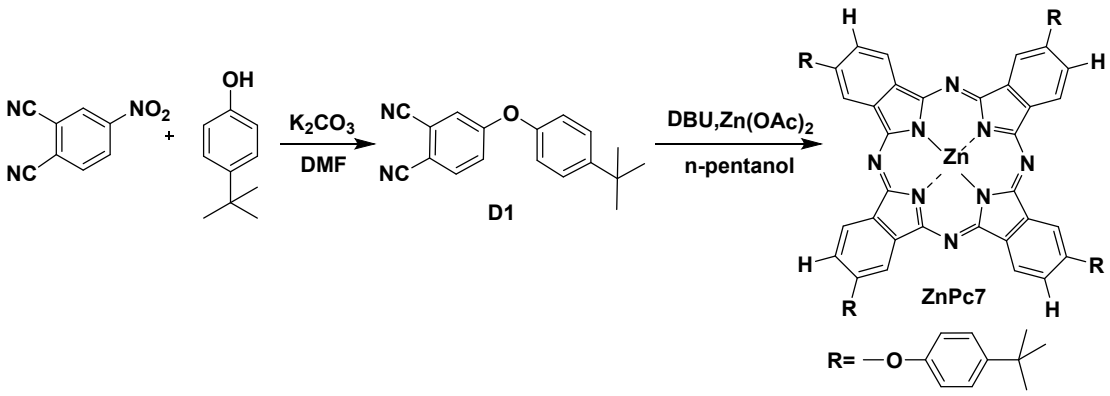


Fig. S4 The synthesis route of ZnPc7.

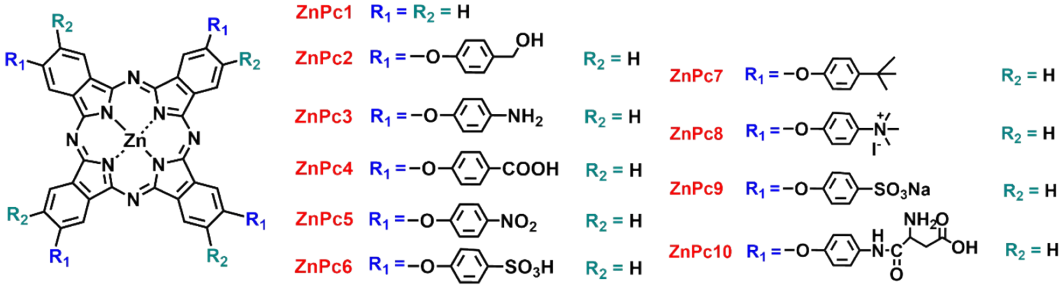


Fig. S5 The structures of ZnPc1-ZnPc10.

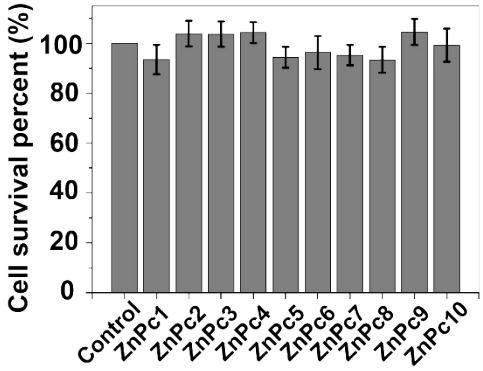
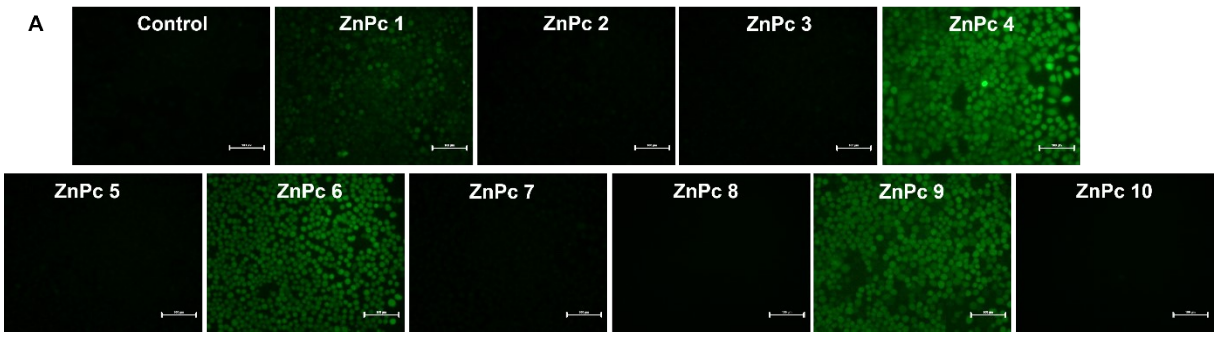


Fig. S6 The dark cytotoxicity of ZnPc1-ZnPc10.



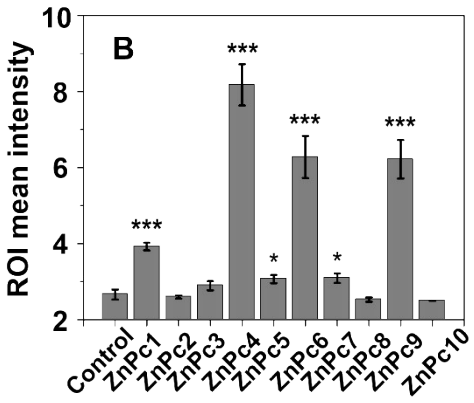


Fig. S7 (A) Fluorescence images and (B) ROI mean intensity of H_2O_2 burst effect in ZnPcs by BES-H_2O_2-Ac probe (bar = 100 μm, the statistical analysis was used by ANOVA, which was expressed as means ± SD: $^{*}P < 0.05$, $^{***}P < 0.001$ ZnPcs treated groups versus control).

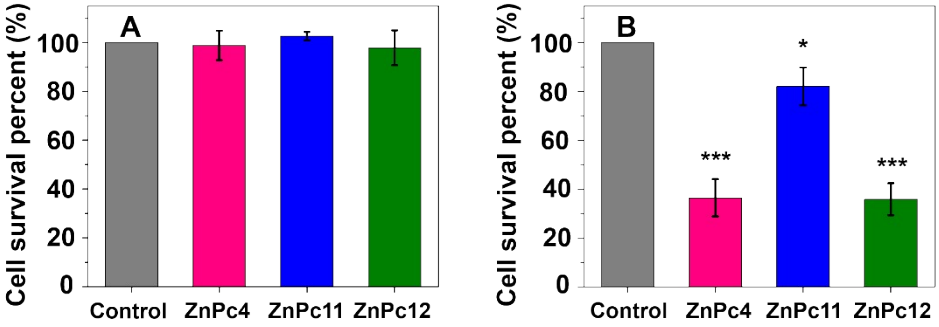


Fig. S8 (A) The dark cytotoxicity of ZnPc4, ZnPc11 and ZnPc12. (B) The light cytotoxicity comparison of ZnPc4, ZnPc11 and ZnPc12 (data was expressed as means ± SD: $^{*}P < 0.05$, $^{***}P < 0.001$ ZnPcs treated groups versus control).

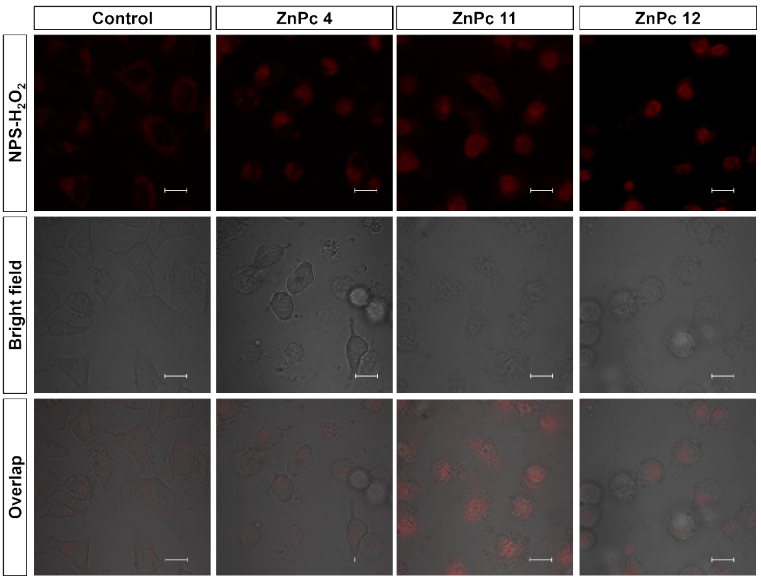


Fig. S9 Fluorescence signal comparison of ZnPc4, ZnPc11 and ZnPc12 by H_2O_2 probe (NPS-H_2O_2).[7, 8]

Histidine is an N containing amino acid. Therefore, the N signal (from N in the histidine) was strongly present in Fe_3O_4 by XPS spectra, which indicated that histidine was successfully modified on the surface of Fe_3O_4 nanoparticles.

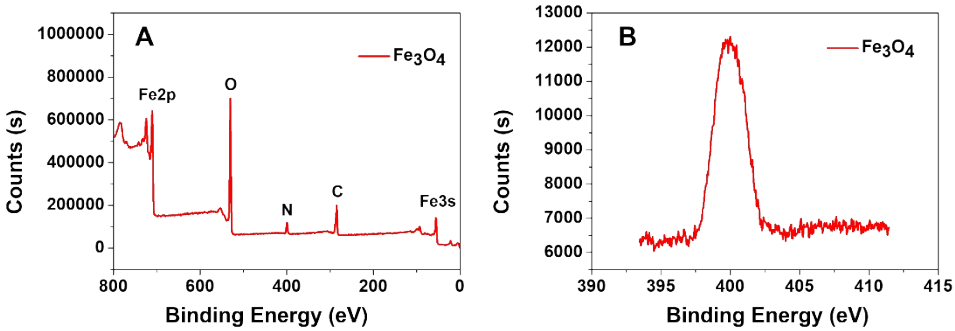


Fig. S10 (A) The XPS patterns of Fe_3O_4. (B) The XPS N signal of Fe_3O_4.

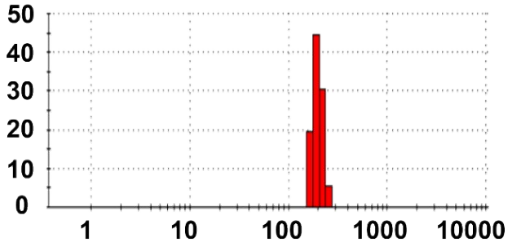
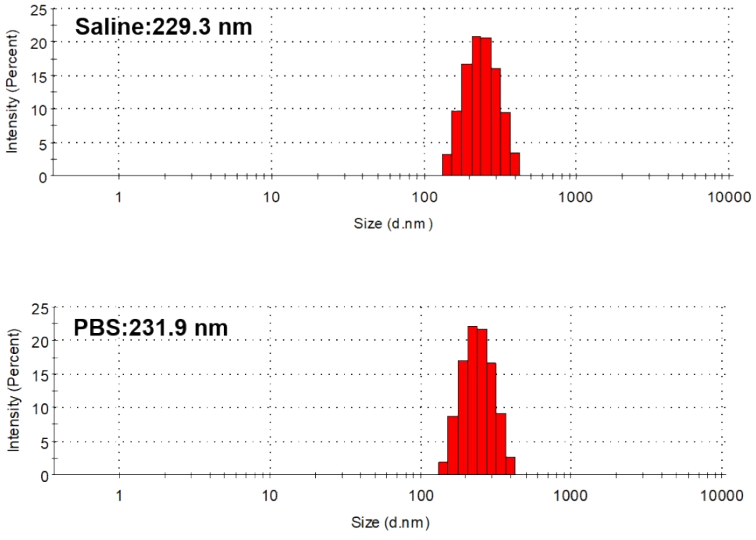


Fig. S11 DLS pattern of HFZP.



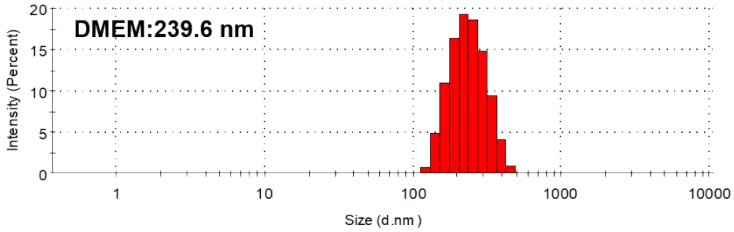


Fig. S12 DLS pattern of HFZP in different culture medium, including saline, PBS, and DMEM.

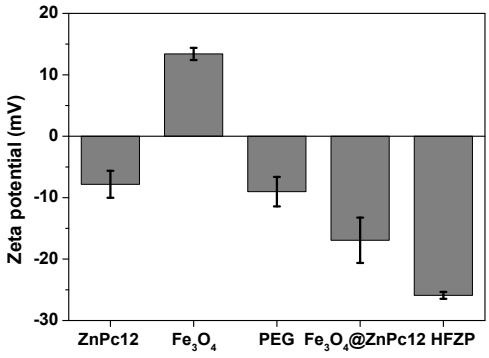


Fig. S13 Zeta potential of ZnPc12, Fe_3O_4, PEG, Fe_3O_4@ ZnPc12 and HFZP.

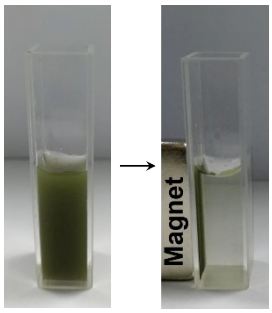


Fig. S14 The dispersion and magnetism property of HFZP.

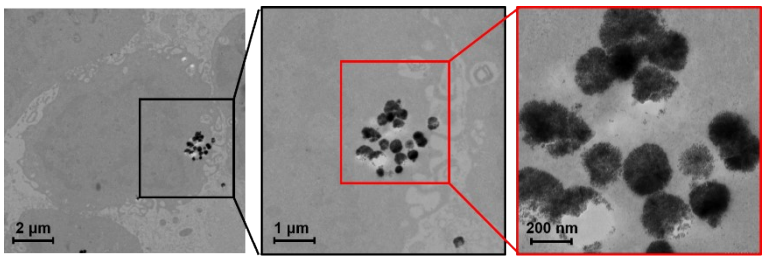


Fig. S15 TEM images of the cells ultrathin section of HFZP.

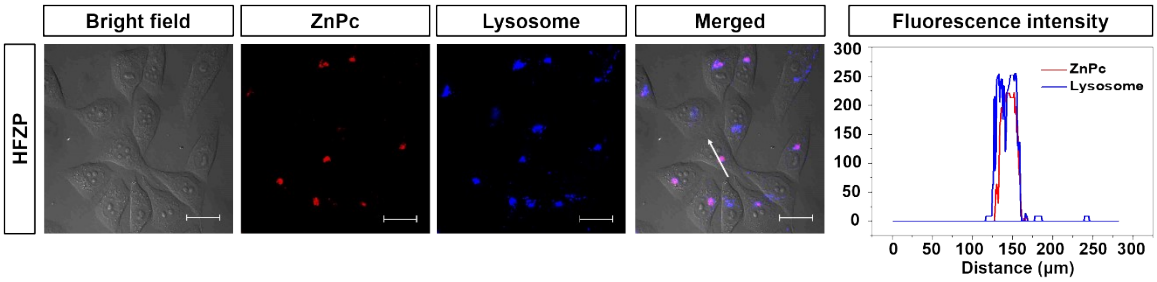


Fig. S16 Subcellular localization of HFZP using Lyso-tracker blue (bar = 20 μm).

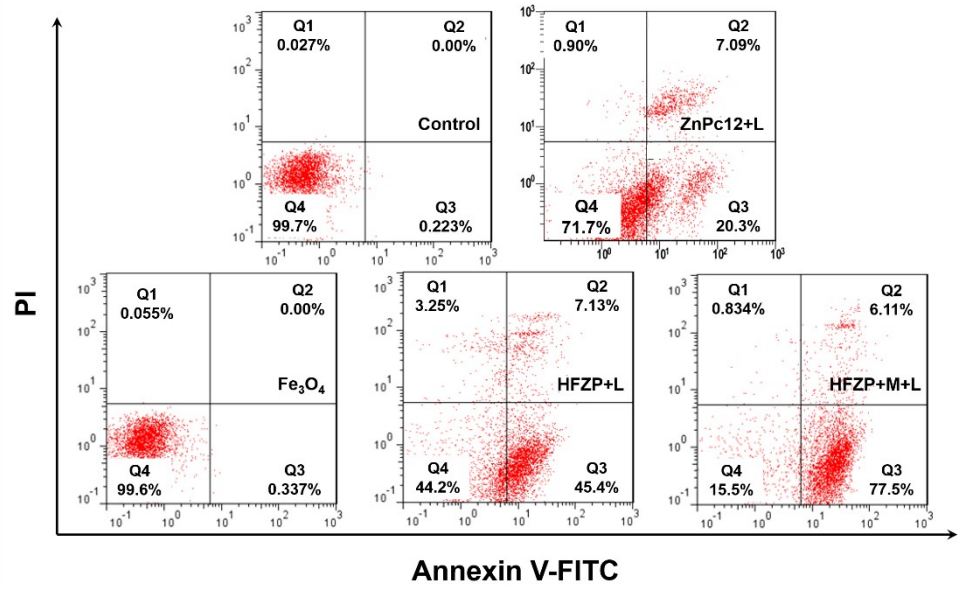
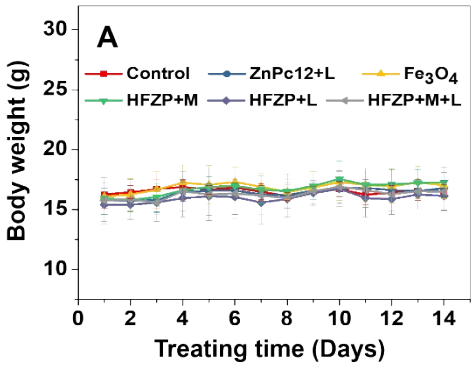


Fig. S17 Cell apoptosis analysis of Control, ZnPc12+L, Fe_3O_4, HFZP+L and HFZP+M+L by FCM after irradiation (5 min) for 24 h (the quadrant of Q4: Annexin V-FITC$^-$, PI$^-$; Q3: Annexin V-FITC$^+$, PI$^-$; Q2: Annexin V-FITC$^+$, PI$^+$ represented the viable, early apoptotic, and late apoptotic cells).



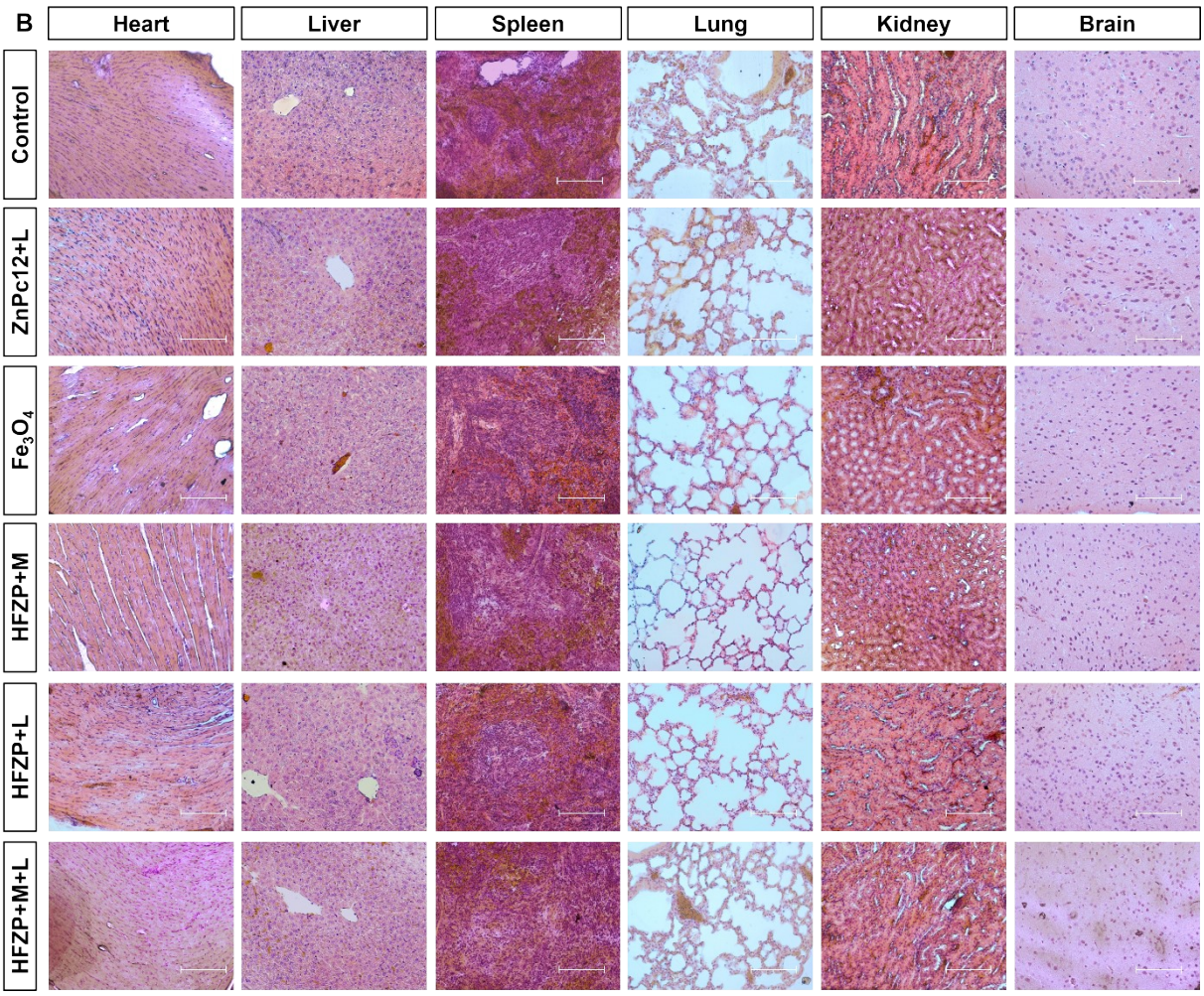


Fig. S18 (A) The weight changes of mice by different drugs treated during 14 days' therapy. (B) H&E staining images of main organs of Control, ZnPc12+L, Fe_3O_4, HFZP+M, HFZP+L, and HFZP+M+L treated mice after 14 days' treatment (bar = 100 μm).

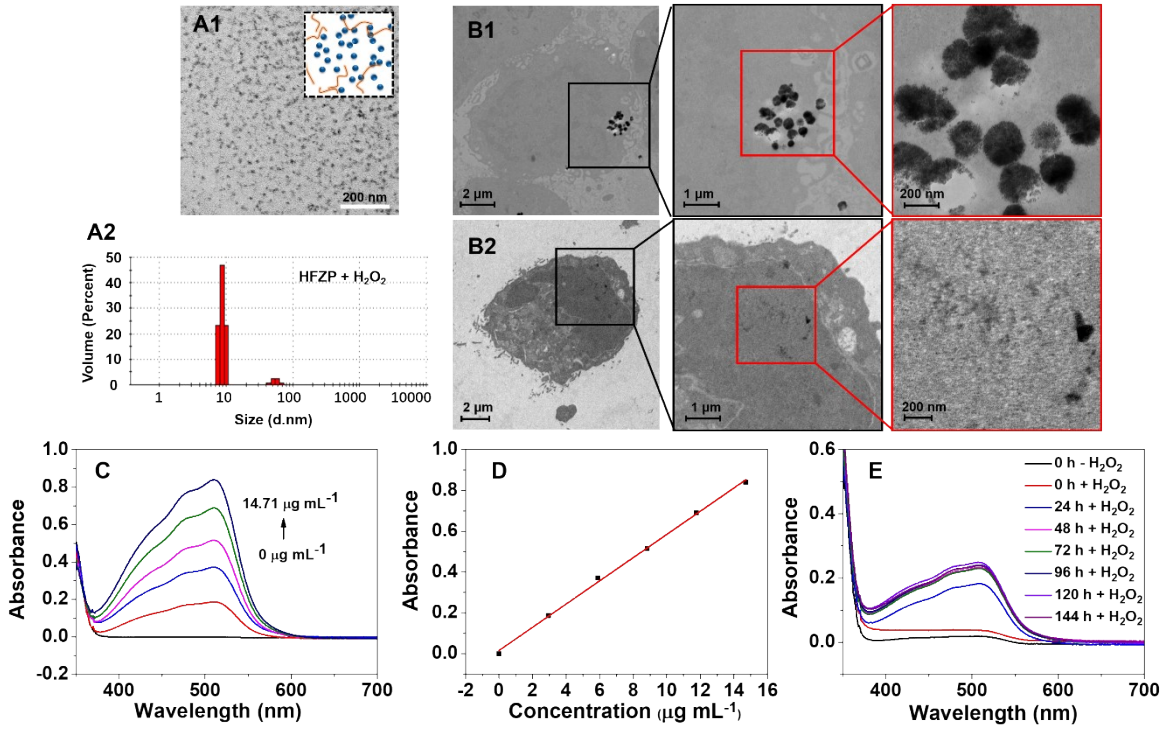


Fig. S19 (A) TEM image and DLS pattern of HFZP in the presence of H_2O_2. (B) TEM images of the cells ultrathin section of HFZP post 4 and 24 h incubation. (C) The absorbance spectrum of different Fe^{2+} concentration with o-phenanthroline to form red complex at 510 nm. (D) The standard curve between the absorbance value at 510 nm and the Fe^{2+} concentration. (E) The absorbance spectrum of Fe^{2+} released from Fe_3O_4 in aqueous solution buffer (pH = 4.5).

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