Electronic Supplementary Material (ESI) for Biomaterials Science.
This journal is © The Royal Society of Chemistry 2023

pH/GSH dual-responsive supramolecular nanomedicine for hypoxia-activated combination therapy

Chang Du,‡[a] Chenwei Wang,‡[c] Shu-Heng Jiang,[a] Xiangqin Zheng,[b] Zelong Li,[b] Yong Yao,[c] Yue Ding*[c], Tingting Chen*[c] and Huan Yi*[b]

a. State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, 200240, China.

b. Department of Gynecology Oncology, Fujian Provincial Maternity and Children's Hospital, Fujian Provincial Key Gynecology Clinical Specialty, The Affiliated Hospital of Fujian Medical University, Zhangzhou, Fujian, China.

c. School of Chemistry and Chemical Engineering, Nantong University, Nantong 226019, P. R. China.

*Corresponding author: yueding@ntu.edu.cn (Yue Ding), ttchen1980@126.com (Tingting Chen), yihuangsrk@126.com (Huan Yi)

Materials

Anhydrous dimethylformamide (DMF, 99.8%, J&K), glucose oxidase (GOD, 65 U/mg, Sigma-Aldrich), tirapazamine (TPZ, 98%, Macklin), trifluoroacetic acid (TFA, 99%, Macklin), amine-modified *β*-CD (Sigma-Aldrich) was used as received. S-(onitrobenzyl)-L-cysteine-N-carboxyanhydride ($_L$-NBC-NCA) monomer were synthesized following the procedure described previously [1]

Synthesis of polymer PEG-Fc

The ferrocene-terminal-modified PEG (PEG-Fc) was synthesized by conjugating ferrocene with PEG-NH_2 through a weakly acidic tumor microenvironment responsive benzoic-imine bond. The 10 mL anhydrous ethanol of PEG-NH_2 (100 mg, 0.019 mmol) was added into 20 mL DMSO solution of formyl-ferrocen (4.01 mg, 0.019 mmol) with vigorous stirring for 12 h. The resulting solution was purified by dialysis against distilled water, followed by freeze-drying to receive production PEG-Fc (76.3 mg, yield: 77.3%). The ^{1}H NMR spectrum of PEG-Fc was shown in **Fig. S2**.

Synthesis of polymer CD-PNBC

The polypeptide CD-PPC was prepared by ring-opening polymerization (ROP). The β-cyclodextrin-terminal-modified poly(ethylene oxide) (CD-PNBC) was synthesized by the ROP of $_L$-NBC-NCA using amine-modified β-cyclodextrin (CD-NH_2) as an initiator in DMF solution at room temperature. Briefly, $_L$-NBC-NCA (141.1 mg, 0.5 mmol) was dissolved in 2.0 mL DMF under N_2 atmosphere, and then a degassed solution of Py-NH_2 (22.7 mg, 0.02 mmol) in DMF was added. The resulting solution was stirred vigorously at room temperature for 48 h and then precipitated dropwise into a large excess of diethyl ether (16 mL). The white precipitate was centrifuged and dried

under vacuum at 35 °C to give 98.9 mg of CD-PNBC (yield: 83.9%). The ^{1}H NMR spectrum of PPC was shown in **Fig. S3**.

Synthesis of polymer CD-PC

The CD-PNBC solution (0.5 mg/mL) in 100 mL of DMF/CH_3CN (v : v = 4 : 1) was irradiated under a high pressure mercury lamp (λ = 365 nm, 150 W) for 12 h to achieve complete photocleavage of NB groups. The solution was concentrated to about 2 mL and then precipitated dropwise into a large excess of ether (16 mL). The brown precipitate was centrifuged and dried under vacuum at 35 °C to give the intermediate product (23.2 mg, 85.7% yield). ^{1}H NMR spectrum of CD-PC was shown in **Fig. S4**.

Fabrication of core cross-linked supramolecular nanomedicine PFC/GOD-TPZ

Generally, PEG-Fc (10 mg), CD-PC (6.03 mg), TPZ (6 mg) and GOD (3.5 mg) were dissolved in DMF (1 mL), and then stirred for 6 h in the dark. Thereafter, distilled water was added into the above solution under vigorous stirring overnight. Then, the mixture solution was oxidized with 0.1 mM H_2O_2 at 37 °C for 4 h to produce the oxidized nanoparticle solution, and then transferred into dialysis tube (MWCO 3500 Da) by dialysis against distilled water for 24 h to give the supramolecular polypeptide nanomedicine PFC/GOD-TPZ. Similarly, the PFC/GOD and PFC/TPZ were fabricated without TPZ and GOD, respectively.

***In vitro* drug release**

The solution of PFC/GOD-TPZ (2.0 mL, 0.5 mg/mL) was placed into dialysis tube (MWCO 3500 Da), followed by dialysis against PBS (20 mL, pH 6.5+GSH, pH 6.5, pH 7.4+GSH or pH 7.4). At selected time intervals, the original dialysate was replaced

with fresh PBS. The collected dialysate was analyzed by UV-vis spectroscopy to calculate the amount of released TPZ.

***In vitro* cytotoxicity**

HeLa cells suspension in DMEM was placed in a 96-well plate at a density of 1×10^4 cells/well (200 μL) and incubated for 24 h. Subsequently, the medium in each well was replaced with fresh medium (pH 6.5 or 7.4) containing PFC/GOD, PFC/TPZ or PFC/GOD-TPZ with different concentrations. After 48 h incubation, the cytotoxicity was analyzed by using MTT assays. As for live/dead assay, the HeLa cells was stained by fluorescein diacetate and propidium iodide, and then imaged by fluorescent microscope.

***In vitro* cell internalization**

The cell internalization of PFC/TPZ or PFC/GOD-TPZ was investigated by confocal laser scanning microscope (CLSM). HeLa cells was seeded into 6-well tissue culture plate at a density of 5.0×10^5 cells/well, and incubated for 24 h. Next, the fresh medium (pH 6.5 or 7.4) containing PFC/TPZ or PFC/GOD-TPZ with same drug concentration (5 μg/mL TPZ equiv.) was added to each well for selected time with or without the 808 nm NIR irradiation (1.0 W/cm^2, 5 min). Then, the medium containing drugs was removed, and the cells were washed with PBS, stained with Hoechst33342 for 15 min, and respectively imaged by CLSM for cell internalization.

***In vivo* parmacokinetics analysis**

The pharmacokinetics analysis was performed using SD rats that were randomly divided into two groups (n = 4). The free TPZ and PFC/GOD-TPZ at equivalent TPZ

concentrations (8 mg/kg) and equivalent GOD concentrations (371 U/kg) were injected via the tail vein. The orbital vein blood (0.3 mL) was obtained at a predetermined time, harvested by centrifugation, and frozen at -20 °C. The TPZ level in blood was measured by fluorescence spectroscopy.

***In vivo* antitumor activity**

The HeLa tumor-bearing mice with a tumor volume (80 mm^3) were spontaneously assigned into five groups (n = 4), and then intravenously injected at 0 day with PBS, PFC, PFC/GOD, PFC/ TPZ or PFC/GOD-TPZ at TPZ equivalent dose of 6 mg/kg and GOD dose of 278.3 U/kg. The tumor volume (V) is calculated according to the following equation: $V = 1/2 \times length \times width^2$. The tumor inhibitory rates (TIR) is calculated by the equation: TIR (%) = 100 × (mean tumor volume of the PBS group - mean tumor volume of others)/(mean tumor volume of the PBS group). At the end of the treatment, all tumors and major organs (heart, liver, spleens, lung, and kidneys) were dissected for histological examination by H&E staining, TUNEL and PCNA assays.

Statistical analysis

The data was expressed as mean ± standard deviations (S.D) using GraphPad Prism software 5.0. The two-tailed analysis of variance and the Student's t-test were used to determine statistical significance. A probability (P) value < 0.01 was indicated to be significant, and $P < 0.001$ was highly significant.

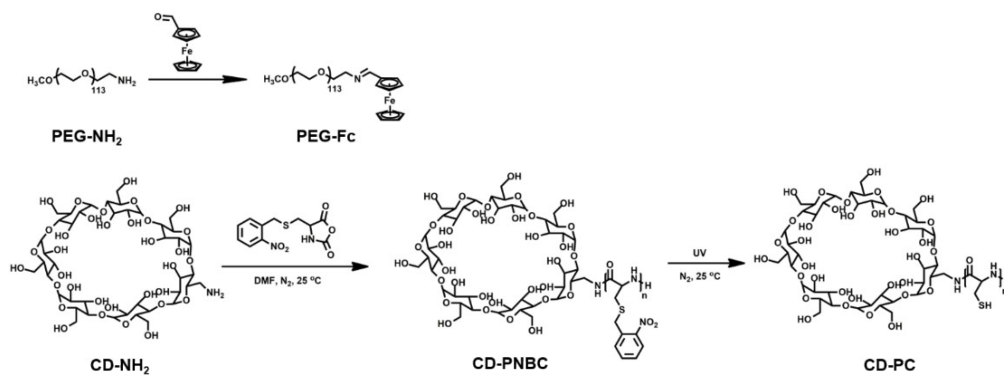

Fig. S1 Synthesis of PEG-Fc and CD-PC.

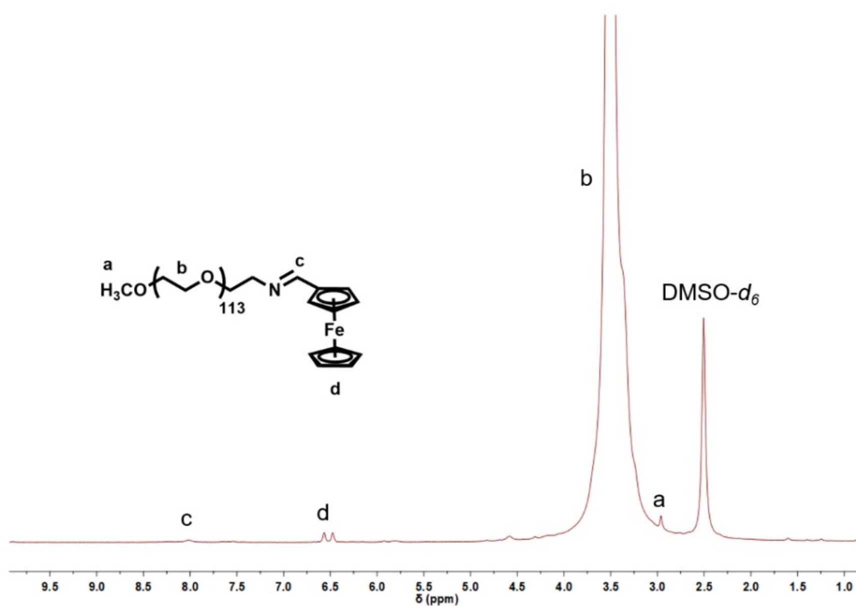

Fig. S2 ^{1}H NMR spectra of PEG-Fc (DMSO-d_6).

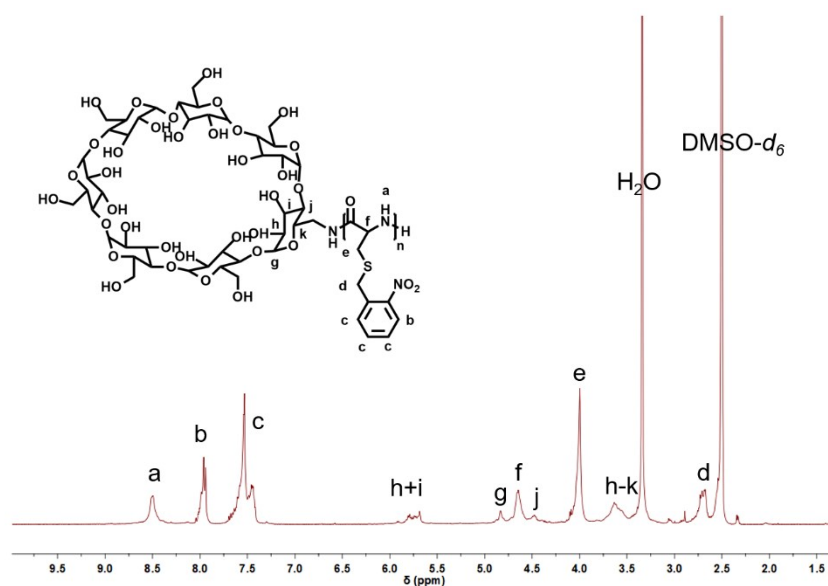

Fig. S3 ^{1}H NMR spectra of CD-PNBC (DMSO-d_6).

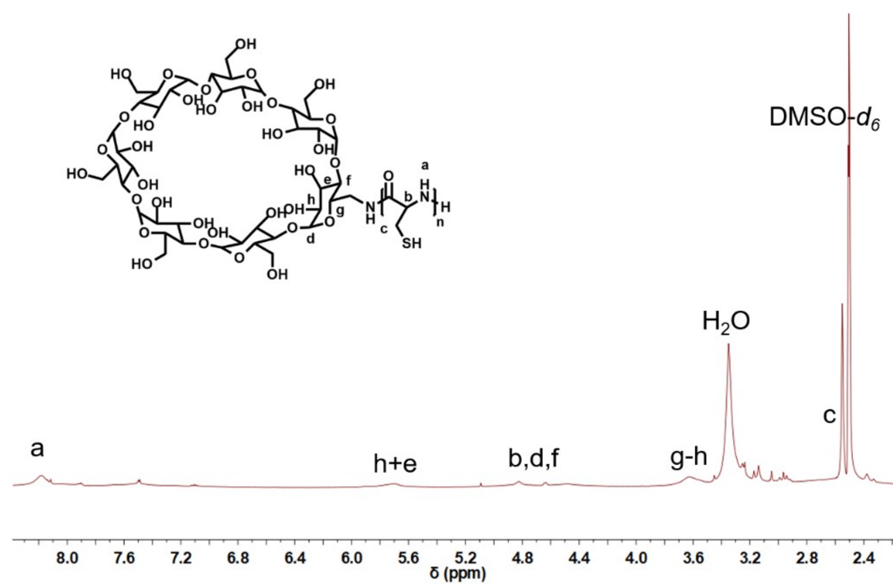

Fig. S4 ^{1}H NMR spectra of CD-PC (DMSO-d_6).

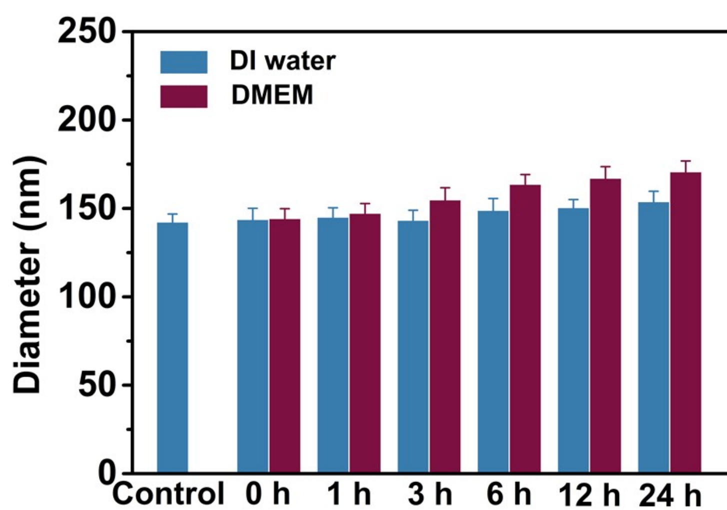

Fig. S5 The dependence of hydrodynamic diameter on incubation time in DI water and DMEM for PFC/GOD-TPZ.

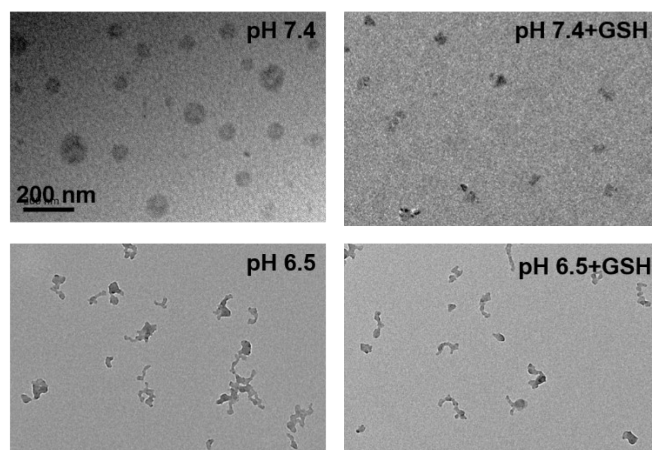

Fig. S6 TEM images of PFC/GOD-TPZ incubated at different conditions.

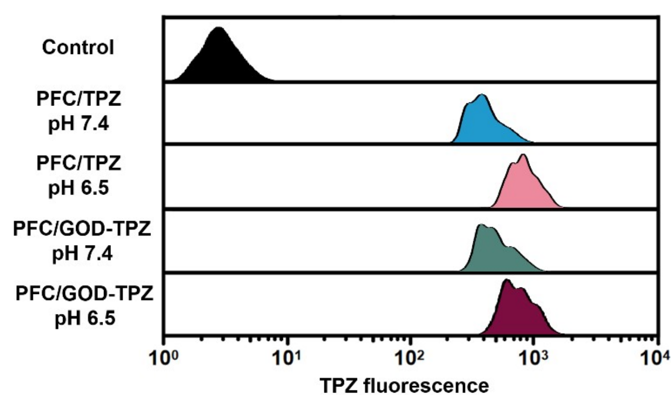

Fig. S7 Flow cytometry histograms of PFC/TPZ and PFC/GOD-TPZ at pH 7.4 and 6.5.

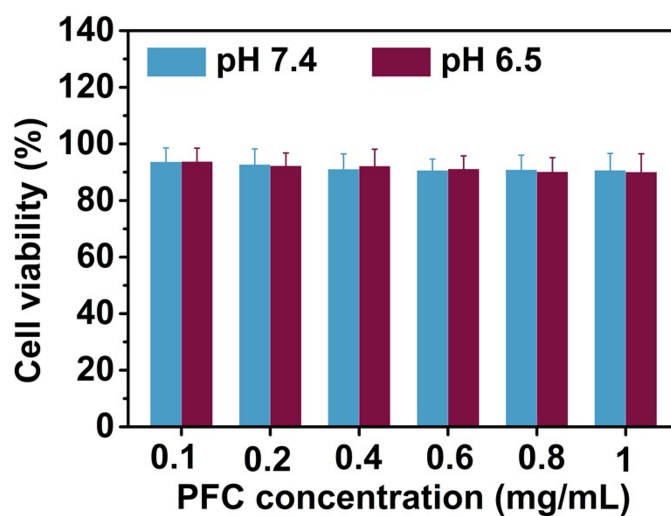

Fig. S8 Cytotoxicity of PFC incubated HeLa cells at pH 7.4 and 6.5.

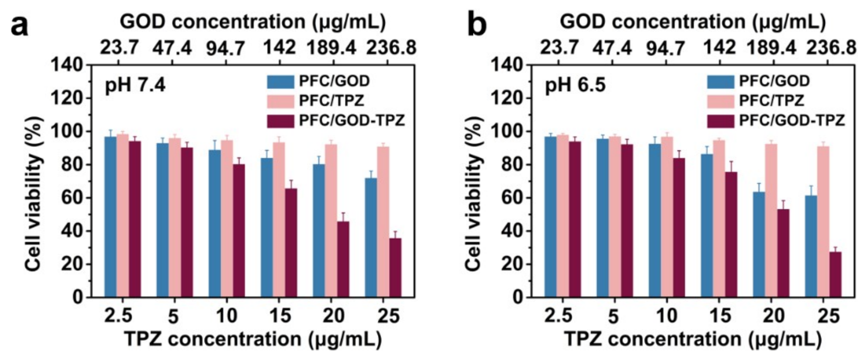

Fig. S9 Cytotoxicity of different samples incubated L929 cells at pH 7.4 (a) and 6.5 (b).

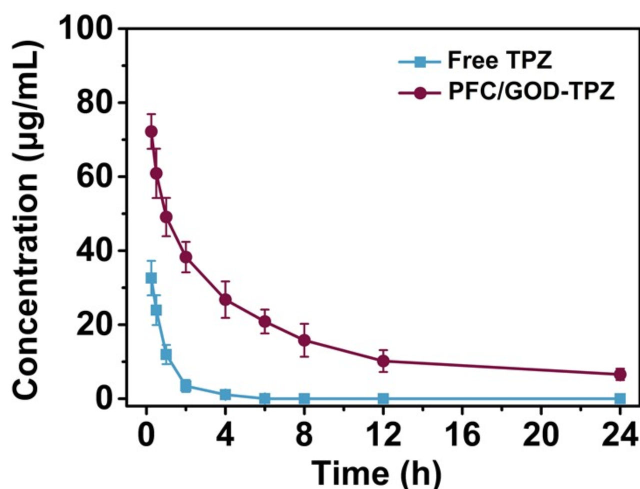

Fig. S10 Representative plasma concentration-time profiles of free TPZ and PFC/GOD-TPZ.

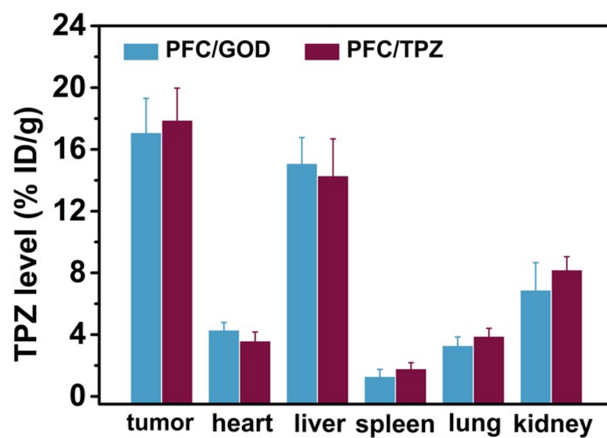

Fig. S11 *Ex vivo* fluorescence quantification of PFC/GOD PFC/TPZ in tumor tissue and major organs at 12 h of post-injection.

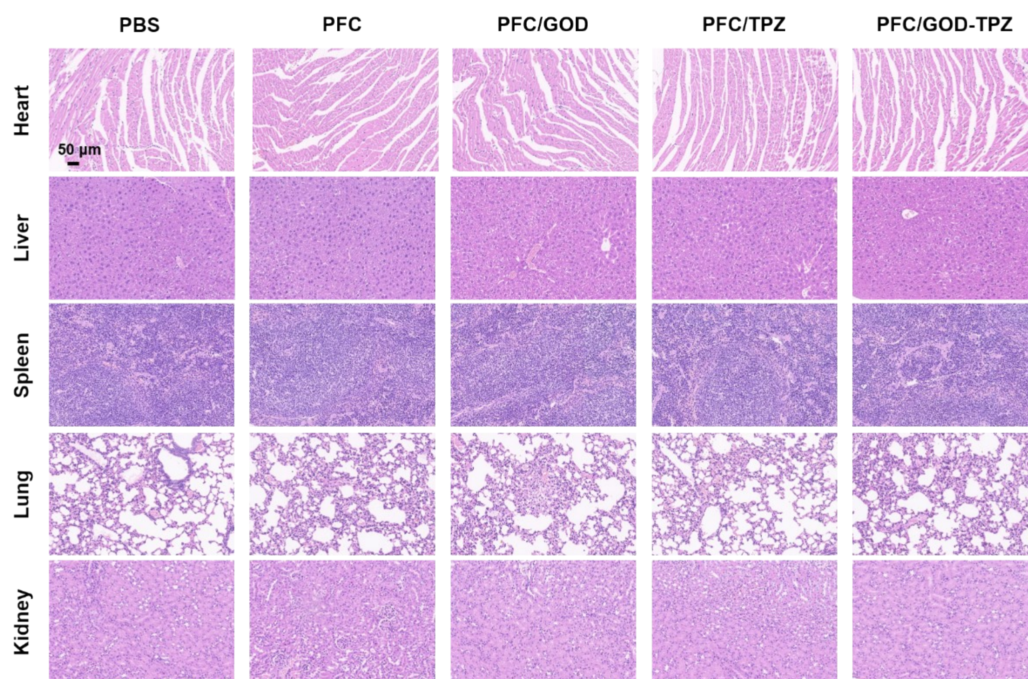

Fig. S12 H&E-stained tissue sections from the major organs (heart, liver, spleen, lung, and kidney) dissected after 18 days treatments (magnification × 400).

Supplementary References

[1] Y. Ding, C. Du, J. Qian, et al., Polym. Chem. 9 (2018) 3488-3498.