Supporting Information for

A Pd nanoparticle-loaded nanoscale Covalent Organic Framework to enhance tumor therapy by synergistic hydrogen/photothermal therapy

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Content

1. Materials and Instrumentations

1,3,5-Tris(4-aminophenyl)benzene and 2,5-bis(2-propyn-1-yloxy)terephthalaldehyde were purchased from Jilin Province Yanshen Technology Co., Ltd. $Pd(NO_3)_2 \cdot 2H_2O$ was purchased from Sahn Chemical Technology (Shanghai) Co., Ltd. Methylene blue (MB) was purchased from TCI (Shanghai) Development Co., Ltd. Benzaldehyde was purchased from Aladdin Reagent Co., Ltd. All reactants were used as purchased without further purification. Acetonitrile, acetic acid, and triethylamine were purchased from Sinopharm Chemical Reagent Co., Ltd. Ultra-pure water was prepared with an Aquapro System (18 MΩ). CCK-8 assay kit was purchased from Dojindo (Shanghai, P.R. China). Phosphate-Buffered Saline (PBS), Dulbecco's Phosphate-Buffered Saline (DPBS), and Fetal bovine serum (FBS) were purchased from VivaCell (Shanghai, P. R. China). Dulbecco's Modified Eagle Medium (DMEM) was purchased from HyClone Laboratories; Normocin was purchased from Invivogen (Thermo Fisher Scientific). Penicillin Streptomycin Mixtures (Pen-Strep), and Trypsin-EDTA Solution (0.25%) were purchased from HyClone Laboratories, Inc. Normocin was purchased from Invivogen (San Diego, CA, USA).

Ultraviolet-visible (UV−vis) absorption spectra were recorded on a Shimadzu UV-2700 Double Beam UV−vis Spectrophotometer. Scanning electron microscopy (SEM) micrographs were recorded on a Hitachi SU8010 Scanning Electron Microscope. Transmission electron microscope (TEM) micrographs were recorded on a Hitachi HT7700 120 kV Compact-Digital Transmission Electron Microscope. Powder X-ray diffraction (PXRD) patterns were obtained on a Rigaku SmartLab SE X-Ray Powder Diffractometer with Cu Kα line focused radiation (λ = 1.5405 Å) from 2θ = 2.00° up to 50.00° with 0.01° increment. Hydrodynamic particle size and zeta potential were measured using Malvern Zetasizer Nano ZS90 System. Laser scanning confocal fluorescence images were captured with a Leica TCS SP8 Confocal Laser Scanning Microscopy with an objective lens (×20). Microplate assays were carried out on a Molecular Devices SpectraMax i3x Multi-Mode Microplate Detection System. Laser scanning confocal fluorescence images of cells were captured with a Leica TCS SP8 Confocal Laser Scanning Microscopy equipped with 405, 458, 488, 514, 561, and 633 nm lasers. Glass bottom dishes and 4-well chamber slides (Cellvis, Mountain View, CA, USA) were used for cell culture to provide biological replicates of each experiment.

2. Cell Culture and Laboratory Animals

The MCF-7 (human breast adenocarcinoma cell line) was provided by Stem Cell Bank, Chinese Academy of Sciences (Shanghai, P. R. China), and cultured in DMEM supplemented with FBS (10%), Normocin (50 μg/mL), penicillin (100 U/mL) and streptomycin (100 μg/mL) in an atmosphere of CO_2 (5 vol%) and air (95 vol%) at 37°C.

Nude mice (BALB/c-nu, femina, aged 4 weeks, 9–12 g) were purchased from Spearfish (Beijing) Biotechnology Co., Ltd. Animal experiments were reviewed and approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China (approval number AEECSDNU2024001). All the animal experiments complied with relevant guidelines of the Chinese government and regulations for the care and use of experimental animals.

3. Synthesis of materials

3.1 Synthesis of COF (1)

1,3,5-Tris(4-aminophenyl)benzene (23.89 mg, 0.068 mmol), 2,5-bis(2-propyn-1-yloxy)terephthalaldehyde (24.70 mg, 0.102 mmol) were dissolved in acetonitrile (25 mL) and glacial acetic acid (2.7 mL) was added and stirred for 12 h at room temperature. The product was centrifuged and washed 3 times with acetonitrile, and washed with ethanol for 3 times. Finally, the solids were dried in air at 40°C.

3.2 Synthesis of Pd@COF (2)

COF (**1**) (10 mg) was dispersed in H_2O (10 mL), and $Pd(NO_3)_2 \cdot 2H_2O$ (8 wt.% in H_2O, 50 μL) was added while stirring. Triethylamine (38 μL) was added to adjust the pH to neutral and stirred for 30 min while passing nitrogen gas. The mixture was irradiated with simulated sunlight under stirring conditions for 2 h. It was centrifuged, washed three times and dried to give **Pd@COF** (**2**) as a black-green powder.

3.3 Synthesis of $PdH_{0.18}$@COF (3)

5 mL H_2O (5 mg of **2** dispersed in it) were injected into a vial of 25 mL and sealed with a rubber stopper. Then, 100 mg $NaBH_4$ were taken in another vial and sealed with a rubber stopper. Two vials were connected with a capillary. A needle was inserted connected with 1mL syringe into the first vial for the connection to the atmosphere. Then, pH = 5 sulphuric acid solution was injected into the second vial containing $NaBH_4$ using a syringe of 2 mL. $NaBH_4$ in the presence of acidic solution can produce hydrogen gas, so that hydrogen gas could slowly drum into the solution. After 30 min, the needle and capillaries were removed, the vial of **$PdH_{0.18}$@COF** (**3**) was sealed and stored in the dark for the further use.

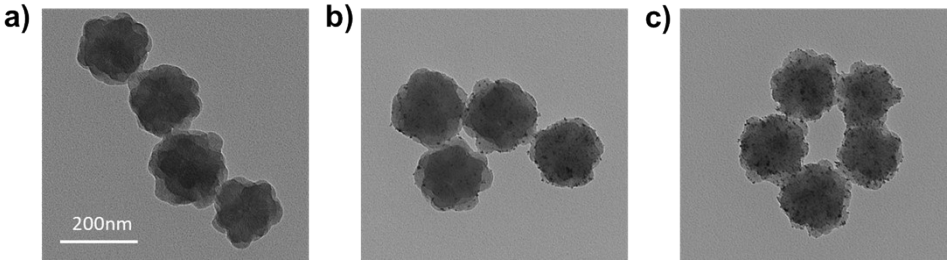

Fig. S1 TEM images of **1**, **2,** and **3**.

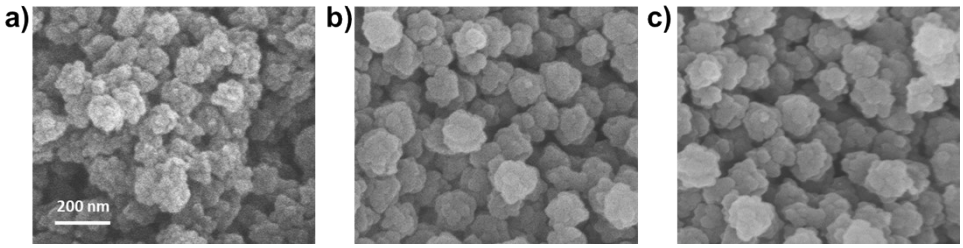

Fig. S2 SEM images of **1**, **2,** and **3**.

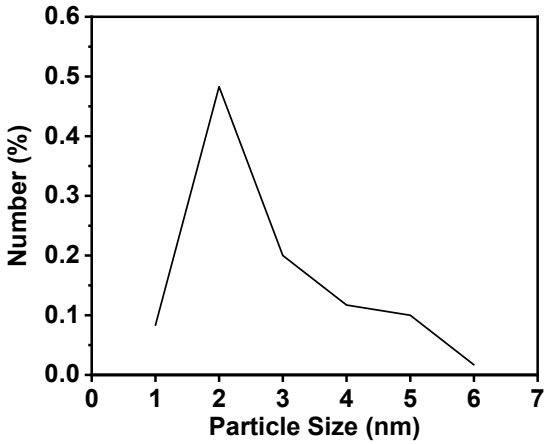

Fig. S3 The statistical diameter distribution of Pd nanoparticles.

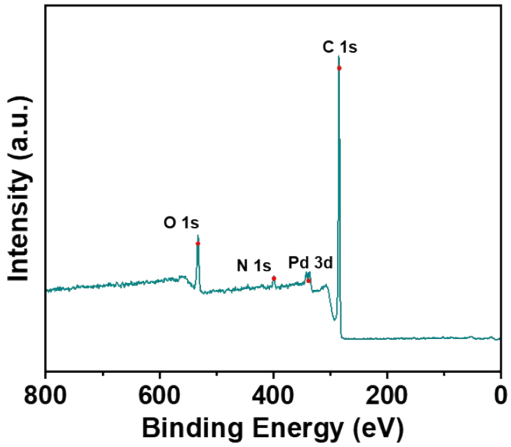

Fig. S4 X-ray photoelectron spectroscopy data of **3**.

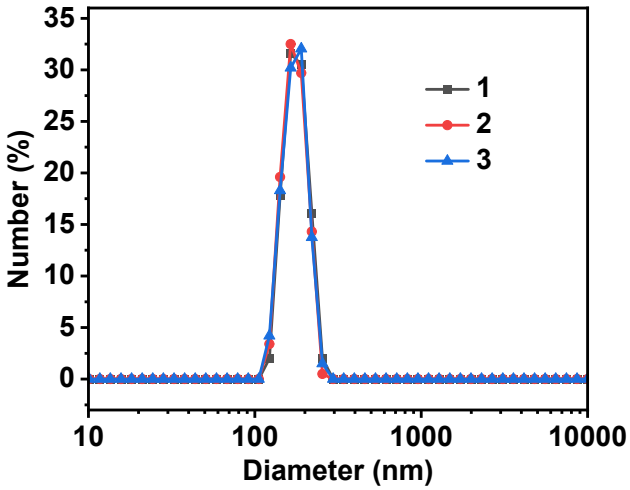

Fig. S5 DLS of **1**, **2,** and **3**.

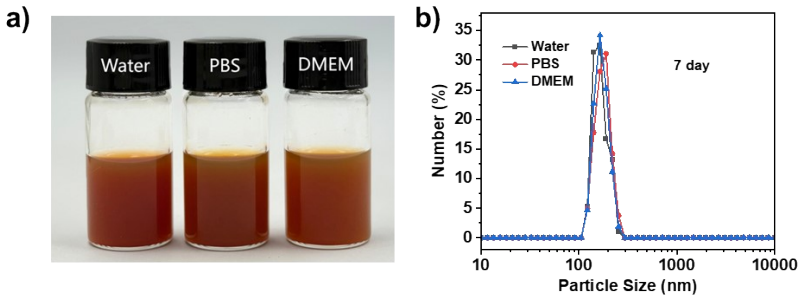

Fig. S6 The stability of **3** in different physiological solutions of H_2O, PBS, and DMEM with 10% fetal bovine serum for 7 days.

4. Measurement of the release of H_2.

MB can be reduced to colorless reduced MB (leucomethylene blue, leucoMB) by hydrogen gas in the presence of catalyst such as platinum or palladium. Based on this principle, MB-Pt reagent usually was served as a probe for determination of hydrogen concentration in water by titration method. We found that zero-valent Pd in **3** nanoparticles have similar catalytic hydrogenation effect as Pt, so a UV-vis spectrum method for in situ quantitative detection of hydrogen release from **3** nanoparticles was established. Because of the linear relation between absorbance of MB at 664 nm and the concentration of hydrogen, the release of reductive hydrogen from **3** can be monitored by real-time detection of the absorbance of MB using UV-VIS spectrometer. The specific measurement steps are described as follows: Firstly, a 1-cm quartz cuvette was filled with 3.4 mL MB solution (15 μM), the solution was bubbled with nitrogen for 30 min to decimate the dissolved oxygen, then the 3 solution (0.1 mL) was gently added and the cuvette was sealed tightly, from this point on, the absorbance of MB was monitored at specific time intervals, until the absorbance at 664 nm keep a constant. It is noticeable that the concentration of MB should not exceed the linearity range of UV-vis, and

should also be high enough to completely react with released hydrogen from **3** nanoparticles. A linear standard curve of absorbance vs concentration of MB at 664 nm wavelength over the range up to 50 μmol/L was plotted. The concentration of reductive hydrogen concentration released was calculated from the final absorbance of MB and the formula of standard curve.

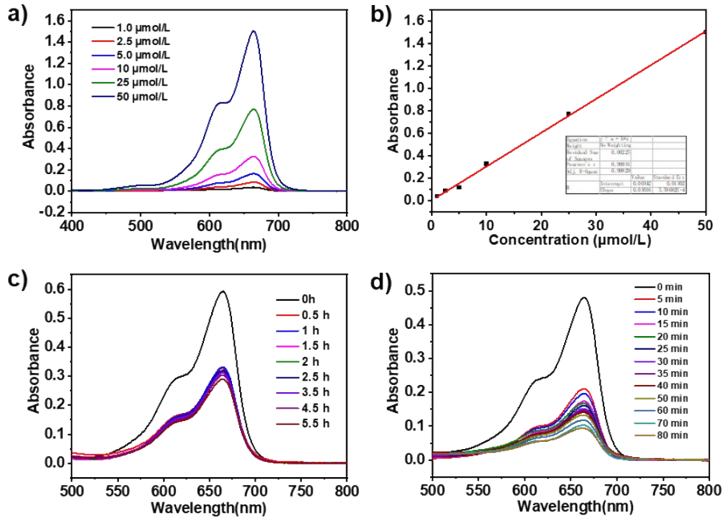

Fig. S7 a) UV-vis absorption spectra of MB aqueous solution at different concentrations. b) The linearly fitted standard curve of MB characteristic absorption intensity at 664 nm vs concentration. c) The time-dependent change of UV- vis absorption spectra of MB after addition hydrogen-enriched water from 0 h to 5.5 h. d) The time-dependent change of UV- vis absorption spectra of MB after addition of **3** at 50°C from 0 min to 80min.

5. Photothermal conversion efficiency

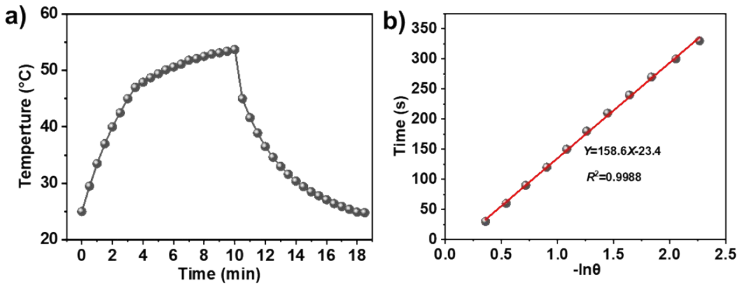

Fig. S8 (a) After 10 min of 808 nm laser irradiation (1.5 W/cm^2) and natural cooling to room temperature, **3** (600 μg/mL) temperature rise was observed. (B) T-(- ln θ) curve of natural cooling period.

The PBS dispersion of **3** (1 mL, 0-600 μg/mL) was added in a quartz dish and irradiated with an 808 nm laser (0.6-1.5 W/cm^2) for 10 min. Then, the laser was turned off to allow the dispersion to cool naturally. The temperature of the dispersion was recorded at 30 s intervals. The photothermal conversion efficiency was calculated according to the following formulas:

$$\eta = \frac{Q_s - Q_w}{I(1 - 10^{-A808})}$$

$$Q_s = hS\Delta T_{s,h}$$

$$Q_w = hS\Delta T_{w,h}$$

$$hS = \frac{mc}{\tau}$$

$$\tau = -\frac{dt}{dln\,\theta}$$

$$\theta = \frac{T_{t,c} - T_{min,c}}{\Delta T_{s,c}}$$

η, photothermal conversion efficiency; A808, the absorption of solution at 808 nm; I, the power of the laser; $\Delta T_{s,h}$, the changed temperature of solution in the heating curve; $\Delta T_{w,h}$, the changed temperature of water in the heating curve; c, specific heat capacity of water; m, solution mass; τ, slope of t - (-ln θ)graph; t, time in the cooling curves; $\Delta T_{s,c}$, the changed temperature of solution in the cooling curve; $T_{min,\,c}$, the final temperature of solution in the cooling curve; $T_{t,c}$, the temperature of solution at different times in the cooling curve. According to the above formula, the photothermal conversion rate is 51.7%.

6. Cell uptake

Cells were seeded into glass-bottom dishes and incubated overnight in a CO_2 incubator. After removal of the culture medium, the cells were incubated with DPBS dispersion of **3** (500 μL, 100 μg/mL) for 2 h in a CO_2 incubator, and washed with DPBS twice carefully. After additional 4 h incubation, cells were washed with DPBS twice. Finally, the laser scanning confocal fluorescence images were captured. The green images of **3** were excited by 488 nm light, and the emission wavelength range was collected at 525±20 nm. Controls were conducted to make sure images were free of crosstalk.

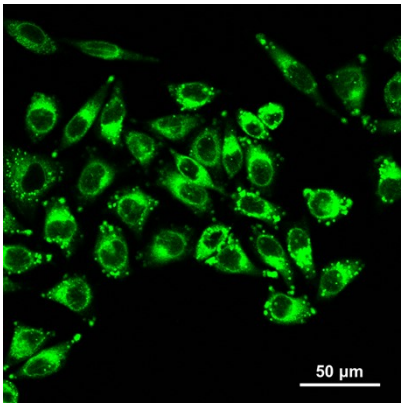

Fig. S9 Cell uptake of **3** in MCF-7 cells.

7. CCK-8 cell viability assay

Cells were seeded into 96-well plates with a cell number of ~5k cells/well and incubated overnight in a CO_2 incubator with 5 vol% CO_2, 37 ℃, and saturated water vapor. After removal of the culture medium, the cells were incubated with DPBS dispersion of **2** (100 μL, 0-400 μg/mL), or **3** (100 μL, 0-400 μg/mL) for 4 h in a CO_2 incubator. For PTT, the cells were exposed to 808 nm laser (1.2 W/cm^2, 10 min). After additional 24 h incubation, CCK-8 (10 μL) and DMEM (100 μL) was added to each well and incubated for additional 3 h in a CO_2 incubator, followed by recording the absorbance at 450 nm.

8. HSP70 content in vitro

To detect the HSP70 relative content in vitro, MCF-7 cells were cultured in Petri dishes overnight, after removal of the culture medium, the cells were incubated with DPBS dispersion of the following 4 groups: (1) **2** (60 μL/mL, 5mL), (2) **2** (60 μL/mL, 5mL) + Light, (3) **3** (60 μL/mL, 5mL), (4) **3** (60 μL/mL, 5mL) + Laser for 4 h in a CO_2 incubator. For laser irradiation groups, 808 nm laser (1.2 W/cm^2) was utilized to irradiate the cells for 10 min. Then all the groups were further cultured for 24 hours in DMEM. SDS-PAGE protein analysis HSP70 relative content.

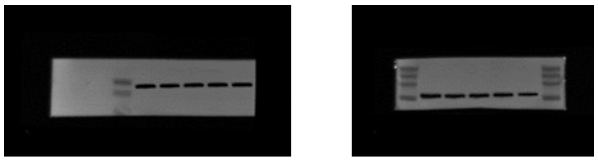

Fig. S10 Unedited original western blot images. (a) HSP70. (b) β-actin.

9. Hemolysis analysis

First, fresh nude mouse blood samples (2 mL) were added to PBS solution (4 mL), and red blood cells (RBC) were separated by centrifugation at 3000 rpm for 10 minutes. After washing 5 times with 10 mL PBS solution, the purified red blood cells were diluted to the original solution with PBS (10 times). For hemolysis assay, 0.2 mL diluted RBCs suspension was mixed with 0.8 mL PBS as a negative control, 0.8 mL deionized water as a positive control, and 0.8 mL **3** suspension at a concentration range of 0 to 500 μg/mL. All mixtures were then allowed to stand at 37 °C for 5 h and centrifuged at 13300 rpm for 10 minutes. The absorbance of 541 nm supernatant was measured by a synergy HT multi-mode microplate reader. The hemolytic percentage of red blood cells was calculated by the following formula.

Hemolysis Rate = [(Dt – Dnc) / (Dpc – Dnc)] ×100%

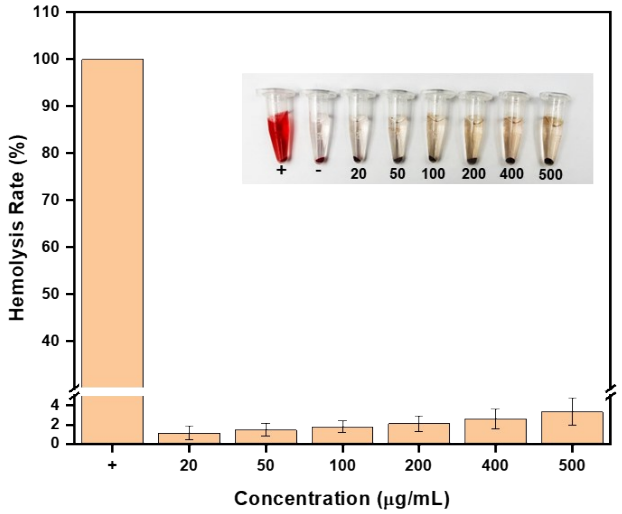

Fig. S11 Hemolytic assay using red blood cells incubated with control solvents and different concentrations of **3**.

10. *In Vivo* Antitumor Therapy

MCF-7 cancer cells (10^6 cells) suspended in DPBS (100 μL) were subcutaneously injected into the flanks of each mouse to establish the MCF-7 xenograft model. The length (L) and width (W) of the tumor were determined by a digital calliper. The tumor volume (V) was calculated by the formula $V = 1/2 \times L \times W^2$. When the tumor size reached ~100 mm^3, the nude mice bearing MCF-7 tumours (n = 25) were randomly distributed into 5 groups, were the control group, **2**, **3**, **2** + light, **3** +light. After intratumoral injection PBS (50 μL), **2** or **3** (50 μL, 1 mg mL^{-1}), the nude mice were fed for 4 h, and for the treatment group, light treatment (808 nm laser, 1.5 W/cm^2, 6 min) was performed on the tumor site. The mice continued to be fed for 14 days. The tumor volume and nude mouse body weight were recorded daily during the experimental period.

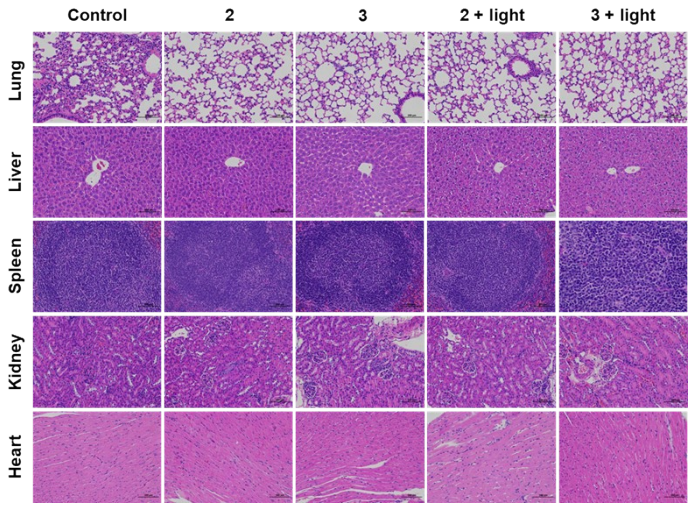

Fig. S12 H&E-stained tissue sections from the heart, liver, spleen, lung, and kidney of the nude mice at the end of the treatment. Scale bar, 100 μm.

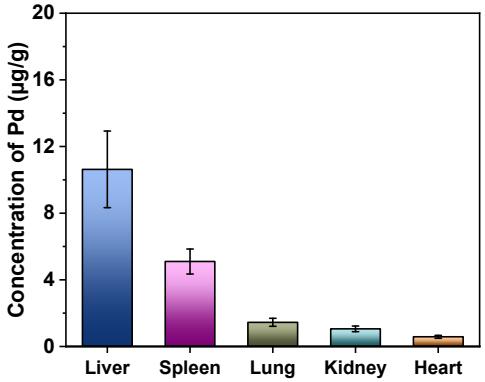

Fig. S13 The in vivo biodistribution of Pd after 72 hours of intratumoural injection of **3** (n=3).