Electronic Supplementary Information (ESI)

for

Two-Photon Fluorescence and MR Bio-imaging of Endogenous H_2O_2 in Tumor Microenvironment by a Dual-Mode Nanoprobe

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1. Reagents and Materials

The citric acid (Alfa Aesar) and branched poly(ethylenimine) (BPEI, M=1800) (Aladdin, Shanghai, China) were purchased for the preparation of TP-CQDs. Potassium permanganate ($KMnO_4$) was purchased from Beijing Chemicals (Beijing, China). 2-(N-morpholino)ethanesulfonic acid (MES) was purchased from Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China). H_2O_2 stimulant phorbol-12-myristate-13-acetate (PMA) and H_2O_2 scavenger N-acetyl-L-cysteine (NAC) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Fetal bovine serum (New Zealand Origine, Corning) was purchased from Sigma-Aldrich (Shanghai, China). Cell Counting Kit-8 (CCK-8) assay was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Ultrapure water was used throughout the whole experiments, which was purified by a Milli-Q reference system (Millipore, USA). All other reagents were of analytical grade (Aladdin, Shanghai China) and used without further purification.

2. Apparatus

FT-IR spectrophotometer (Thermo Nicolet 360) was used to attain the Fourier transform infrared (FT-IR) spectra. High-resolution transmission electron microscope (TEM, JEM-2100F, 200kV) was employed for the morphological characterization of the nanomaterials. RF-6000 fluorescence spectrophotometer (Shimadzu, Japan) was obtained for fluorescence spectrograms. Shimadzu UV-1800 spectrophotometer (Shimadzu, Japan) was acquired for absorbance measurements. The cell and tissue fluorescence images were recorded by a fluorescence microscope (Leica, Germany and Olympus, Japan). The measuring of longitudinal relaxation time (T_1) was obtained by MesoMR23-060H-I NMR analyzing and imaging system (Suzhou Neimai Electronic Technology Co., LTD, Suzhou, China).

3. Synthetic procedure of TP-CQDs

The TP-CQDs were synthesized from the mixture of CA and BPEI by the reported pyrolysis method.[1-4]

4. Synthetic procedure of TP-CQDs@MnO_2

TP-CQDs@MnO_2 was synthesized by a one-pot procedure. Briefly, 250 μL of MES buffer (0.1 M, pH 6.0) was respectively added to a 1.5 mL Eppendorf microtube (EP tube) containing the TP-CQDs solution (250.0 μL, 375.0 $\mu g \cdot mL^{-1}$). Then adding 200.0 μL 10.0 mM $KMnO_4$ into the EP tube filled with the above mixture. Next, the corresponding volume of deionized water was added to make the final volume of the mixture 1.0 mL. Ultrasound the resulting homogeneous mixed solution for 30 minutes until the solution turned brown. Subsequently, TP-CQDs@MnO_2 were collected by centrifugation (12000 rpm) and washed three times with deionized water.

5. Synthetic procedure of MnO_2 Nanosheets

MnO_2 nanosheets were synthesized similar to TP-CQDs@MnO_2 without TP-CQDs.

6. Detection of H_2O_2

Normally, stock solution was prepared by phosphate buffered saline solution (PBS, 150.0 mM, pH 5.0) and stored at room temperature, which containing different concentrations of H_2O_2. In the experiment of gradually releasing fluorescence signal in response to different concentrations of H_2O_2, the pre-prepared with different concentrations of acidic H_2O_2 with adding the original prepared TP-CQDs@MnO_2 (375.0 μg·mL^{-1}). The mixture was oscillated at room temperature and the fluorescence signal was detected by a fluorescence spectrophotometer, the excitation wavelength of TP-CQDs at 352.0 nm.

7. Response of TP-CQDs@MnO_2 as MRI Contrast Agents to H_2O_2.

The T_1-weighted signals were detected for different concentrations of Mn^{2+} of TP-CQDs@MnO_2, which were treated with different concentrations of acidic H_2O_2. The Mn^{2+} were measured at 0.5 T on a MesoMR23-060H-I. In detail, TP-CQDs@MnO_2 (3.0 mg·mL^{-1}) was prepared in deionized water, the total (1.0 mL) consists of TP-CQDs@MnO_2 (63.0 μg·mL^{-1}) with different concentrations of acidic H_2O_2. The mixture was incubated at room temperature for 0.5 h before the MR test.

8. Specificity Investigations

In order to assess the selectivity of the pro-prepared TP-CQDs@MnO_2 for acidic buffer solution, the selectivity of agents including the common metal cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+}), common anions (Cl^-, ClO^-, NO_3^-, SO_4^{2-}, CH_3COO^-, $C_6H_5O_7^{3-}$, $S_2O_8^{2-}$), amino acids [L-cysteine (L-Cys), L-tryptophan (L-Try)], macromolecule (Glucose, GO), 10% FBS,[5] 100.0 μM glutathione (GSH),[6] 50.0 μM ascorbic acid (AA)[6] and 1.0 mM micromolecule (H_2O_2).

9. Interference Detection

To evaluate the interference of TP-CQDs@MnO_2 for acidic buffer solution containing H_2O_2, other interfering agents including the common metal cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+}), common anions (Cl^-, ClO^-, NO_3^-, SO_4^{2-}, CH_3COO^-, $C_6H_5O_7^{3-}$, $S_2O_8^{2-}$), amino acids [L-cysteine (L-Cys), L-tryptophan (L-Try)], macromolecule (Glucose, GO), ascorbic acid (AA), 100.0 μM glutathione (GSH) and 10% FBS in 1.0 mM acidic H_2O_2 that were studied under fluorescence modes.

10. Cell Culture

Immediately prior to the fluorescence imaging experiments, an amount of 0.4 mL of MCF-7 cells (8.0×10^3 mL^{-1}) was seeded into a 24-well plate and allowed the adhesion in a 5.0% CO_2 incubator at 37.0 °C overnight. After that, the overnight culture medium was replaced by 0.4 mL of new culture medium which contained TP-CQDs@MnO_2 (375.0 μg·mL^{-1}). After 3 h, the mixture medium was discarded and washed with dulbecco's phosphate-buffered Saline (DPBS) for three times, and then incubated with high K^+ buffer (30.0 mM NaCl, 120.0 mM KCl, 1.0 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 5.0 mM glucose, 20.0 mM HEPES) with pH 5.0 buffer in the presence of 10.0 μM nigericin for 3 h at 37.0 °C for OP and TP

fluorescence imaging.

11. Cell Viability Assay

MCF-7 cells were imaged by Leica TCS SP8 confocal laser scanning microscope with 63x oil objective and excited at 405.0 nm for OP images. And the TP images were obtained by TP electrophysiological microscope (Olympus FV1200 MPE) with 40x water objective and excited at 750.0 nm.

12. OP and TP Fluorescence Imaging of MCF-7 cells

In vitro cytotoxicity of the nanoprobe TP-CQDs@MnO_2 was evaluated using Cell Counting Kit-8 (CCK-8) assay in MCF-7 cells. Briefly, 100.0 μL of cells (8.0×10^3 mL^{-1}) were seeded into a 96-well plate and allowed the adhesion in a 5.0% CO_2 incubator at 37.0 °C. After that, the overnight culture medium was replaced by 100.0 μL of new culture medium which contained various concentrations of TP-CQDs@MnO_2 nanocomposite (0, 67.5, 125.0, 250.0, and 500.0 $\mu g\cdot mL^{-1}$) After the different concentrations of TP-CQDs@MnO_2 nanocomposite incubation for 24 h and 48 h, the mixed medium was replaced by 90.0 μL of new medium and 10.0 μL of CCK-8, which were incubated for another 4 h and then the absorbance at 450.0 nm were determined by an enzyme marker.

13. Preparation of Liver Tissue Slices for OP and TP Fluorescence Imaging

Liver tissue was collected for OP and TP imaging. Liver slices were prepared from mouse liver, and the slices were cut into 600.0 mm thickness. After incubation with TP-CQDs@MnO_2 about three hours, and then reacted with different pH values buffer, the control group was treated with DPBS. The liver tissues were imaged by Leica TCS SP8 confocal laser scanning microscope with 20x water objective and excited at 405.0 nm for OP images. And the TP images were obtained by TP electrophysiological microscope (Olympus FV1200 MPE) with 20x water objective and excited at 750.0 nm.

14. MR Imaging of Intracellular different pH in living cells

To explore the MR imaging of MCF-7 cells, 5.0×10^6 cells were seeded into a 10 cm^2 cell culture dish with 10.0 mL medium, and allowed the adhesion in a 5.0% CO_2 incubator at 37.0 °C. After that, the overnight culture medium was replaced by 10.0 mL new culture medium contained with 375.0 $\mu g\bullet mL^{-1}$ TP-CQDs@MnO_2. After incubation for 3 h, the mixed medium was removed and DPBS was used for cleaning three times. Using Trypsin-EDTA (0.25%) to digest and then added 1.0 mL of medium to stop digestion. Cells that had been incubated with the probe were collected and centrifuged to remove the upper solution. Added 2.5 mL medium and made cells evenly distributed in the medium. Added 0.5 mL of above cells into each sterilized EP tube, then 0.5 mL different pH buffer solution were added and incubated in the culture. And then, the upper solution was removed by centrifugation. The last, added DPBS at constant volume (1.0 mL). The MR imaging of MCF-7 cells were obtained by MesoMR23-060H-I (23.4 MHZ, 0.5 T).

15. Quenching mechanism for the quenching of TP-CQDs by MnO_2 nanosheets

The modified Stern-Volmer equation $F_0/(F_0 - F) = 1/f_a + 1/(K_{sv}[C])$ was used to deal with the fluorescence data and analysis the quenching mechanism of TP-CQDs by MnO_2 nanosheets.[7]

F_0: The fluorescence intensity of TP-CQDs without quencher.

F: The fluorescence intensity of TP-CQDs with different concentrations of quencher.

f_a: The fraction of fluorescence quenching.

K_{sv}: The Stern - Volmer quenching constant.

[C]: The concentration of $KMnO_4$ (or MnO_2 nanosheets).

As shown in Fig. S10, there was a good linear relationship between $F_0/(F_0 - F)$ and 1/[C], and the K_{sv} value (7.89×10^3 mol/L) could be obtained from the Stern-Volmer equation was $y = 1.2678x + 0.1335$ ($R^2 = 0.9901$).

The K_q represent dynamic fluorescence quenching rate constant, which reflects the effect of the inter-diffusion and inter-collision in the system on the decay rate of fluorescence molecular lifetime. $K_q = K_{sv}/\tau_0$, τ_0 represent average fluorescent lifetime of fluorescence molecule without quencher. From fluorescence decay curve (Fig. S11), the average fluorescent lifetime of TP-CQDs was calculated 4.83 ns. And $K_q = K_{sv}/\tau_0 = 7.89\times10^3/(4.83\times10^{-9})$ $mol\cdot L^{-1}\cdot s^{-1} = 1.63\times10^{12}$ $mol\cdot L^{-1}\cdot s^{-1}$, that is far greater than 2×10^{10} $mol\cdot L^{-1}\cdot s^{-1}$ (the maximum dynamic quenching constant).[7] Therefore, the fluorescence quenching of TP-CQDs was not only dynamic quenching, but also the static quenching process. And the static quenching was the main effect on the fluorescence quenching of the TP-CQDs by MnO_2 nanosheets.

16. Limit of detection concentration of H_2O_2

As shown in Fig. 1b, and the linear fitting equation was $y = 521.2x + 4431.8$, $R^2 = 0.987$. And then, the LOD can be calculated. The calculation steps were as follows:

1. $y = 521.2x + 4431.8$, $R^2 = 0.987$

   $k = 521.2$, $\sigma = 26.7$ (Without H_2O_2, the mean standard deviation of fluorescence intensity of the nanoprobe TP-CQDs@MnO_2.)

2. $\log LOD = 3\sigma/ k = 3 \times 26.7 / 521.2 = 0.1537$
3. LOD = 1.425 pM

17. Supplementary Schemes and Figures

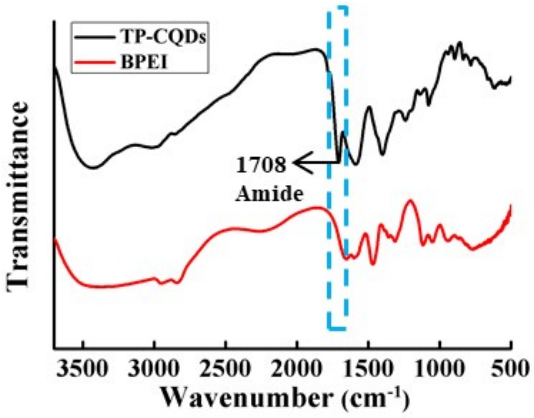

Fig. S1. Fourier Transform Infrared Spectrometer (FT-IR) spectrogram of TP-CQDs and BPEI.

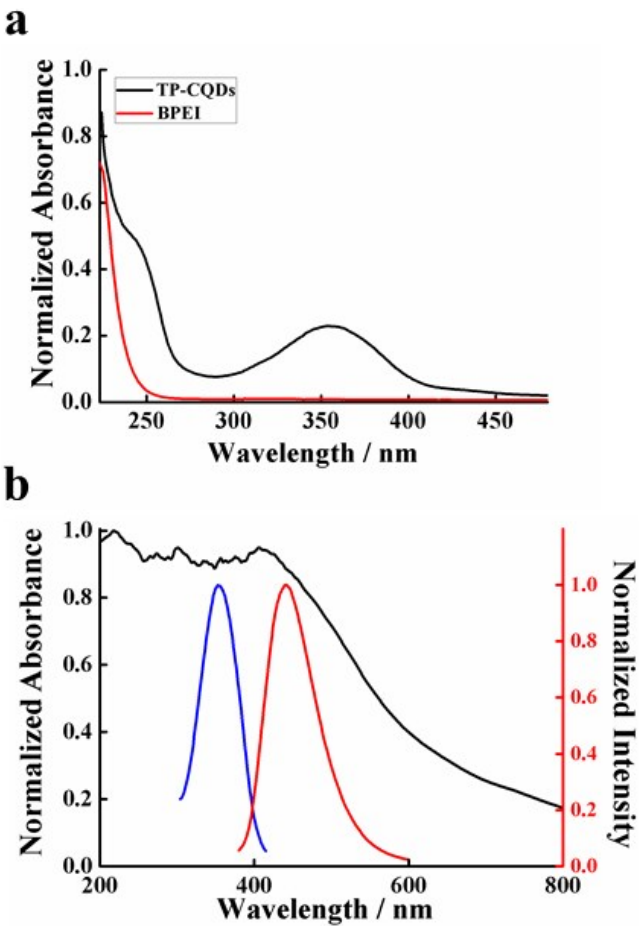

Fig. S2. (a) UV−Vis absorption spectra of TP-CQDs and BPEI. (b) UV−Vis absorption spectrogram of the MnO_2 nanosheets (black line), the fluorescence spectrogram of prepared TP-CQDs: the excitation (blue line) and emission (red line).

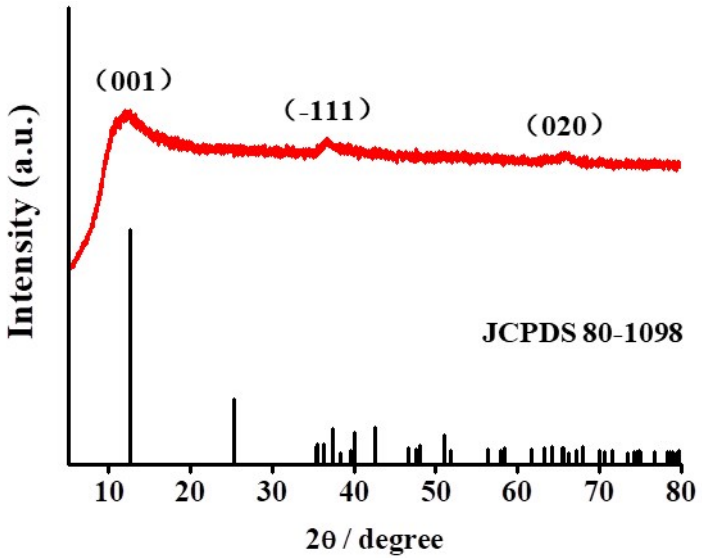

Fig. S3. X-ray powder diffraction (XRD) of MnO_2 nanosheets.

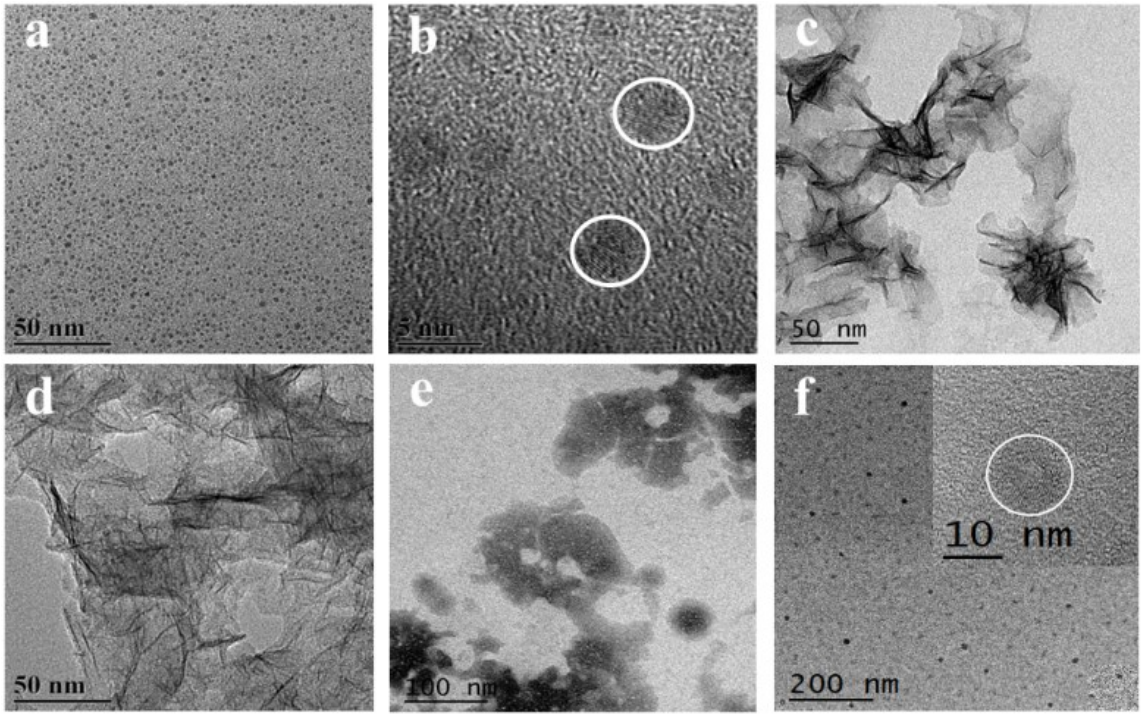

Fig. S4. TEM images of TP-CQDs, MnO_2 nanosheets and TP-CQDs@MnO_2. TEM images of TP-CQDs (a, b), MnO_2 nanosheets (c), TP-CQDs@MnO_2 (d), TP-CQDs@MnO_2 reacted with acidic solution without (e) or with (f) H_2O_2.

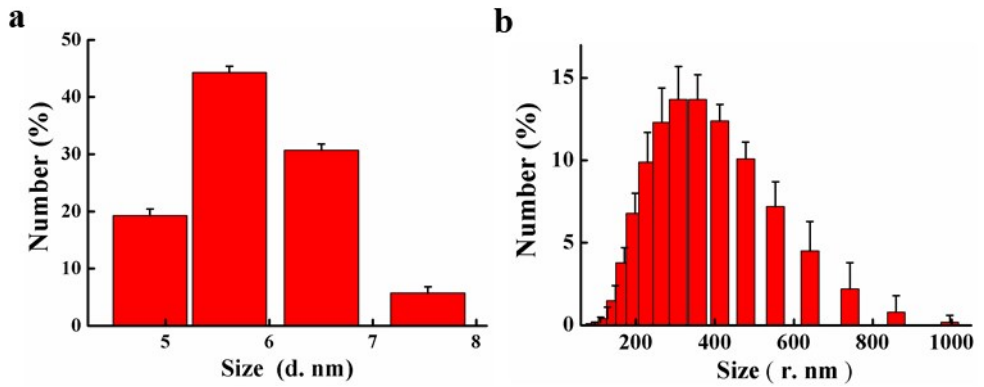

Fig. S5. Particle sizes distribution of TP-CQDs (a) and MnO_2 nanosheets (b) was determined based on dynamic light scattering.

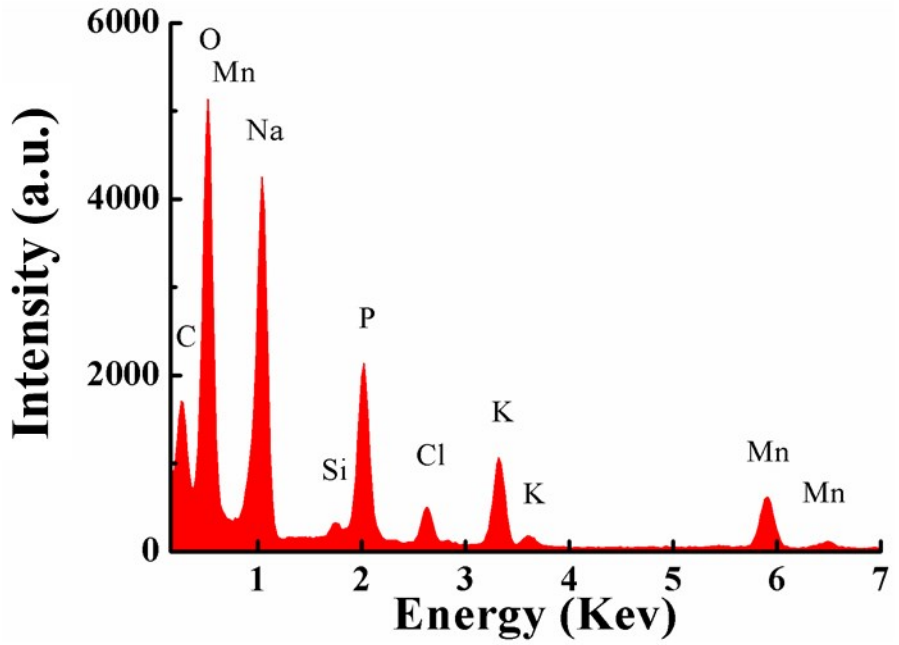

Fig. S6. Energy-dispersive X-ray (EDX) spectrogram of TP-CQDs@MnO_2.

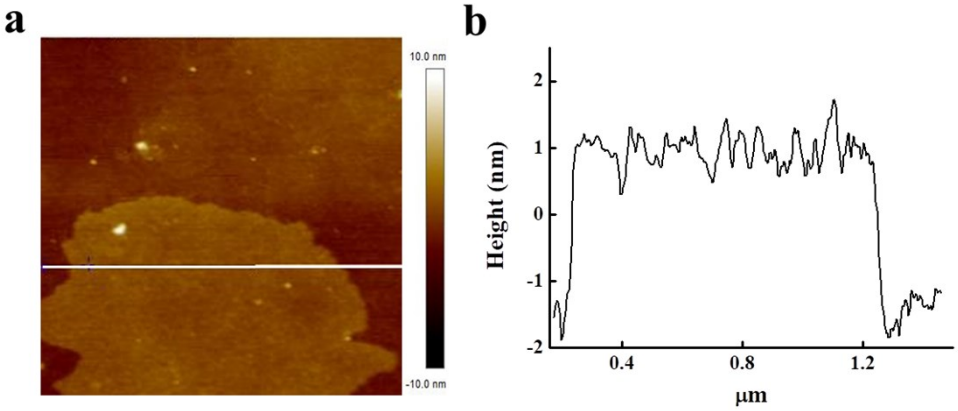

Fig. S7. Atomic force microscopy (AFM) of TP-CQDs@MnO_2.

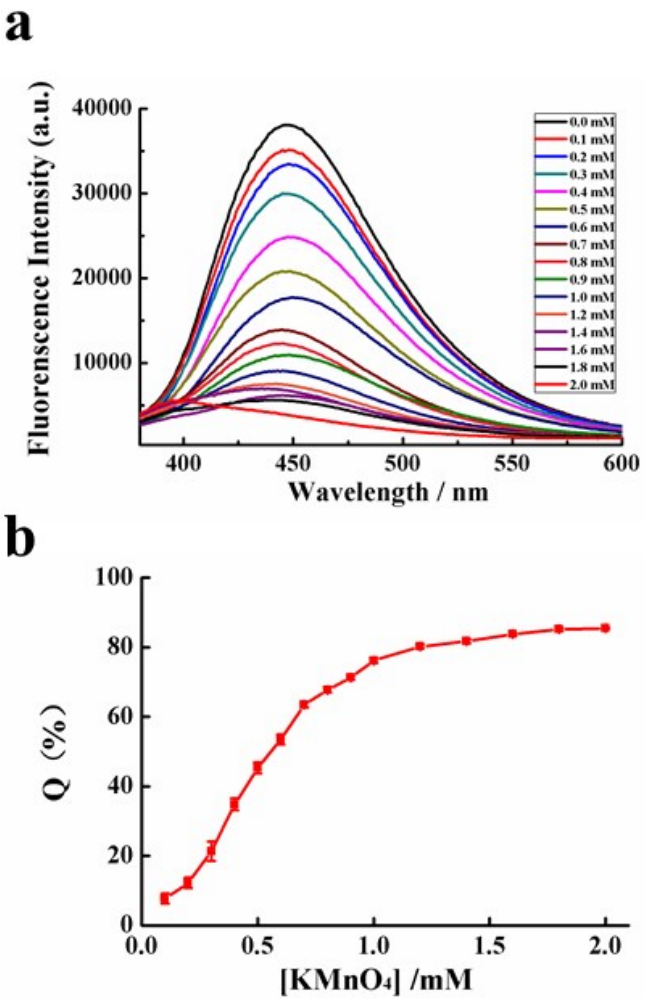

Fig. S8. (a) Fluorescence quenching experiment of TP-CQDs (12.45 μg•mL^{-1}) under different concentrations of $KMnO_4$. (b) Fluorescence quenching rate (Q) versus different concentrations of $KMnO_4$ (0-2.0 mM).

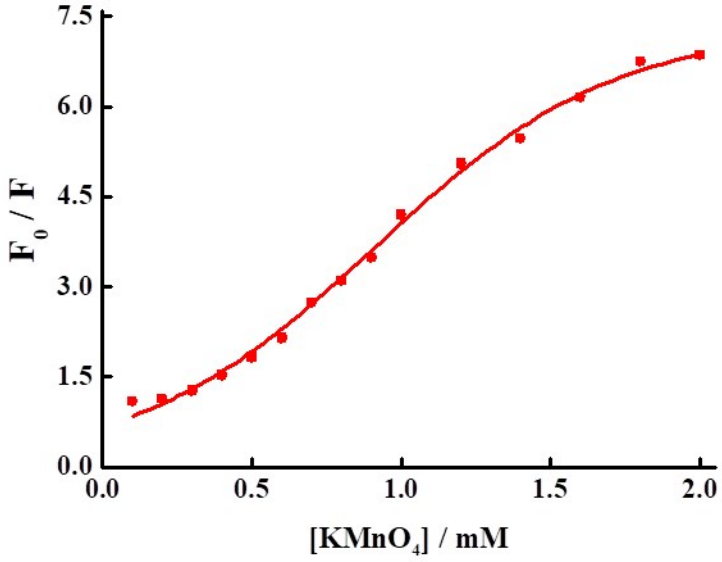

Fig. S9. Stern - Volmer plots of F_0/F versus [$KMnO_4$] in water solvent.

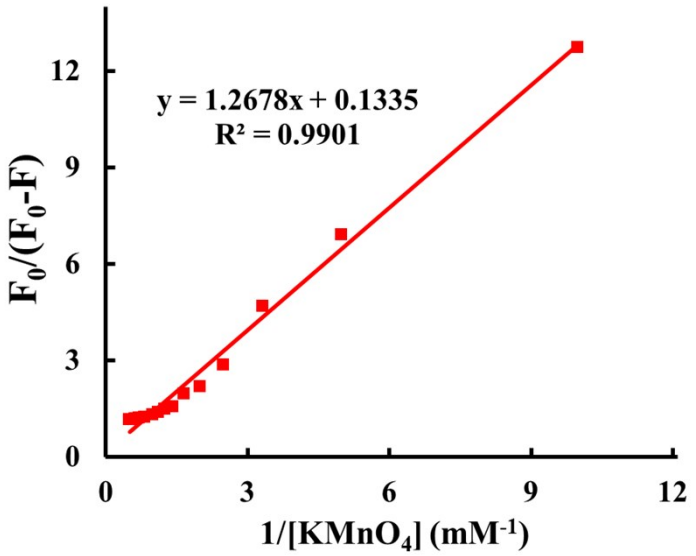

Fig. S10. Modified Stern - Volmer curve of $F_0/(F_0 - F)$ versus the reciprocal of the concentration of $KMnO_4$.

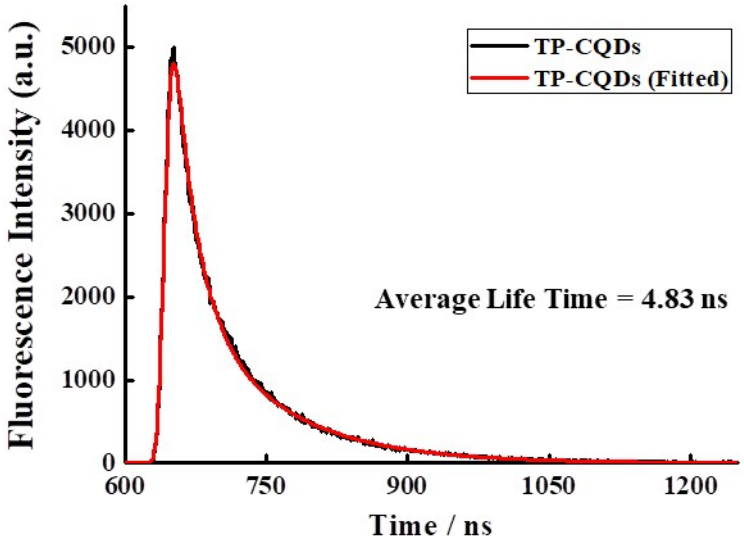

Fig. S11. Fluorescence intensity decay profile of TP-CQDs (black line), the fitted decay profile of TP-CQDs (red line).

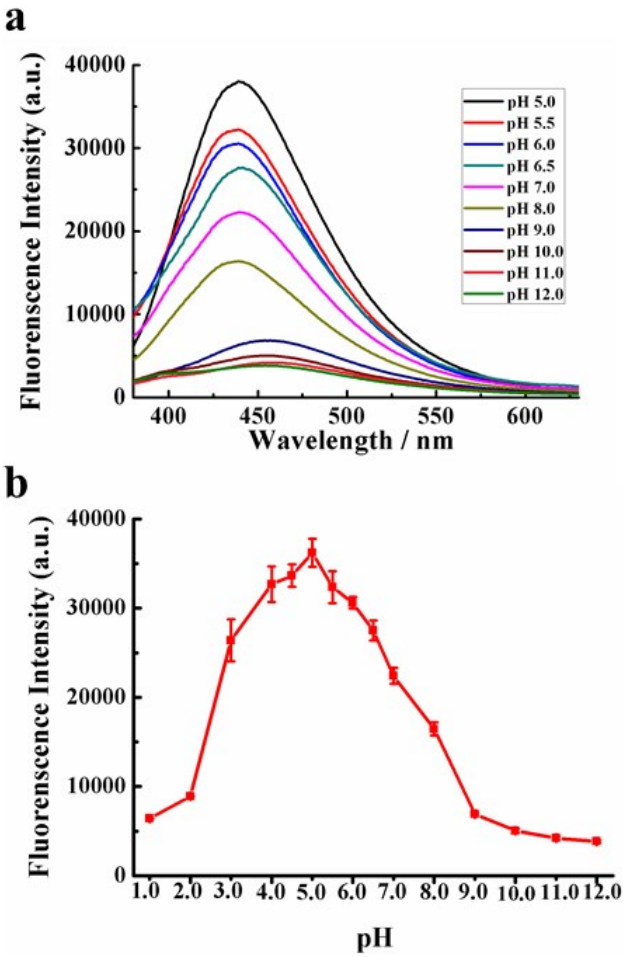

Fig. S12. (a) Fluorescence response of TP-CQDs@MnO_2 solution (375.0 μg•mL^{-1}) with 1.0 mM H_2O_2 under the different pH values from 5.0 to 12.0. (b) Fluorescence response curve of TP-CQDs@MnO_2 (375.0 μg•mL^{-1}) solution under the different pHs from 1.0 to 12.0.

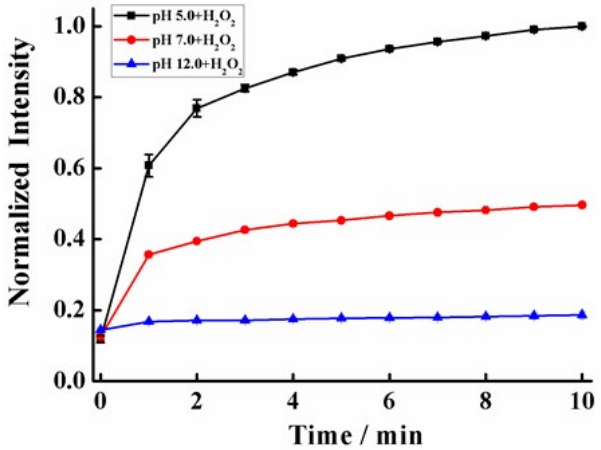

Fig. S13. Fluorescence recovery time curve of TP-CQDs@MnO_2 under different pH values (5.0, 7.0 and 12.0) with H_2O_2.

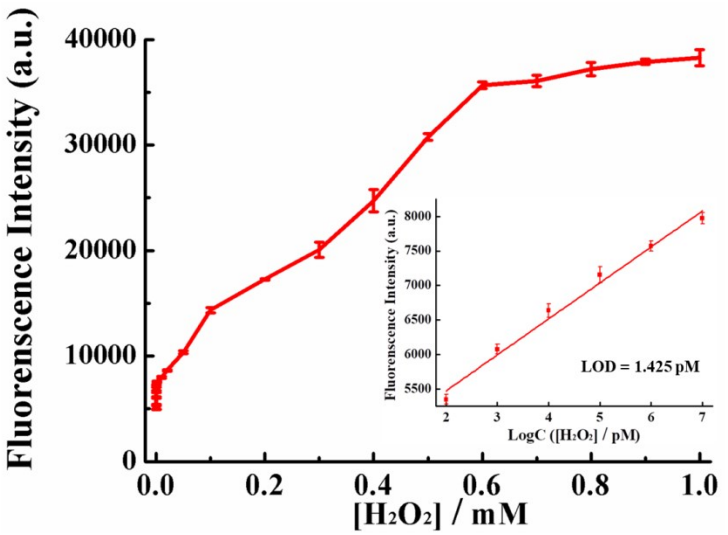

Fig. S14. Fluorescence sensing titration curve of TP-CQDs@MnO_2 solution (375.0 μg•mL^{-1}) contained different concentrations of H_2O_2 under acid condition.

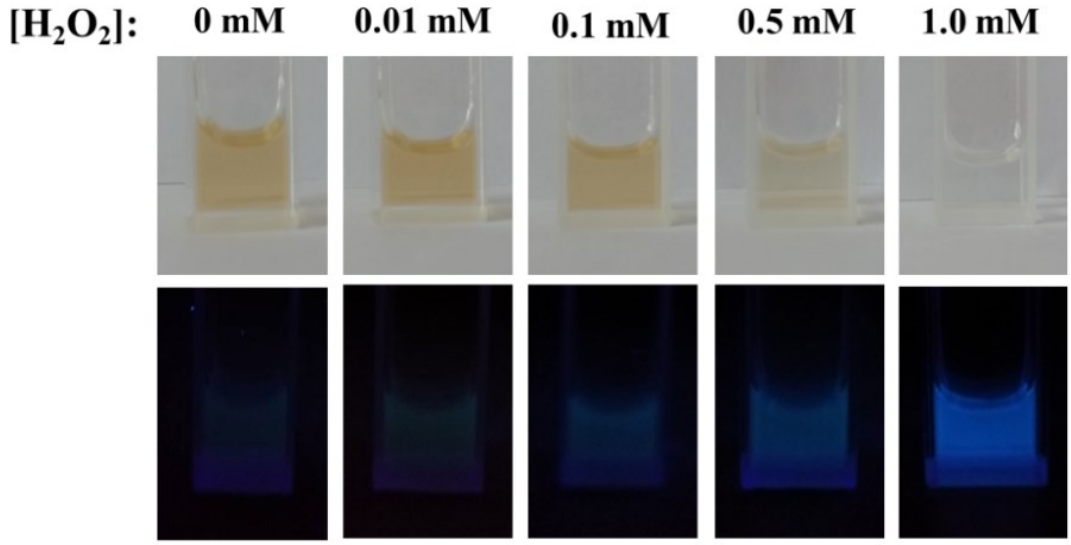

Fig. S15. The digital and ultraviolet photograph of TP-CQDs@MnO_2 (375.0 μg•mL^{-1}) reacted with different concentrations of H_2O_2 under acidic PBS buffer solution.

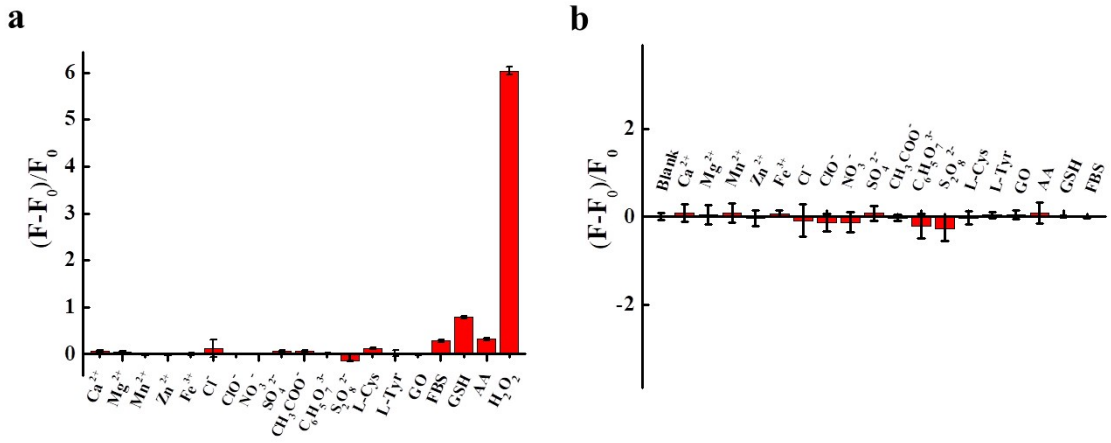

Fig. S16. (a) The selectivity of fluorescence responses of TP-CQDs@MnO_2 to 1.0 mM of Ca^{2+}, Mg^{2+}, 200.0 μM of Mn^{2+}, Zn^{2+}, Fe^{3+}, 250.0 μM of Cl^-, ClO^-, NO_3^-, SO_4^{2-}, CH_3COO^-, $C_6H_5O_7^{3-}$, $S_2O_8^{2-}$, amino acids [L-cysteine (L-Cys), L-tryptophan (L-Try)], macromolecule (Glucose, GO), 10% FBS, 100.0 μM glutathione (GSH), 50.0 μM ascorbic acid (AA) and 1.0 mM H_2O_2 in acidic buffer. (b) The interference of fluorescence responses of TP-CQDs@MnO_2 to 1.0 mM of Ca^{2+}, Mg^{2+}, 200.0 μM of Mn^{2+}, Zn^{2+}, Fe^{3+}, 250.0 μM of Cl^-, ClO^-, NO_3^-, SO_4^{2-}, CH_3COO^-, $C_6H_5O_7^{3-}$, $S_2O_8^{2-}$, amino acids [L-cysteine (L-Cys), L-tryptophan (L-Try)], Glucose (GO), ascorbic acid (AA), 100.0 μM glutathione (GSH) and 10% FBS in 1.0 mM acidic H_2O_2. λ_{ex}=352.0 nm.

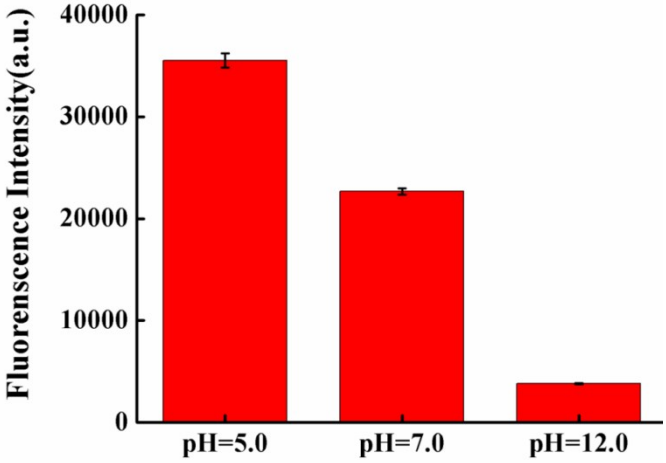

Fig. S17. The PBS buffer solutions with 1.0 mM H_2O_2 under different pH values of PBS buffer solution was prepared from Yongjiang River to explore the stability of TP-CQDs@MnO_2 (375.0 μg•mL^{-1}) in complex environments.

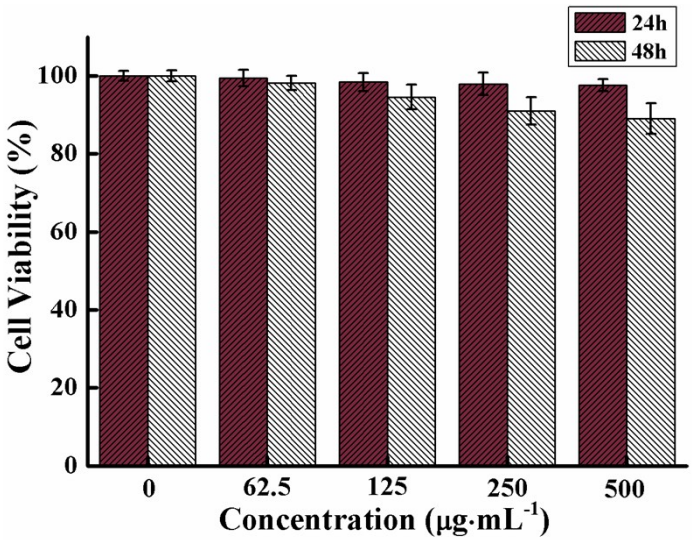

Fig. S18. Cell viability of MCF-7 cells after being incubated with different concentrations of TP-CQDs@MnO_2 for 24 h and 48 h.

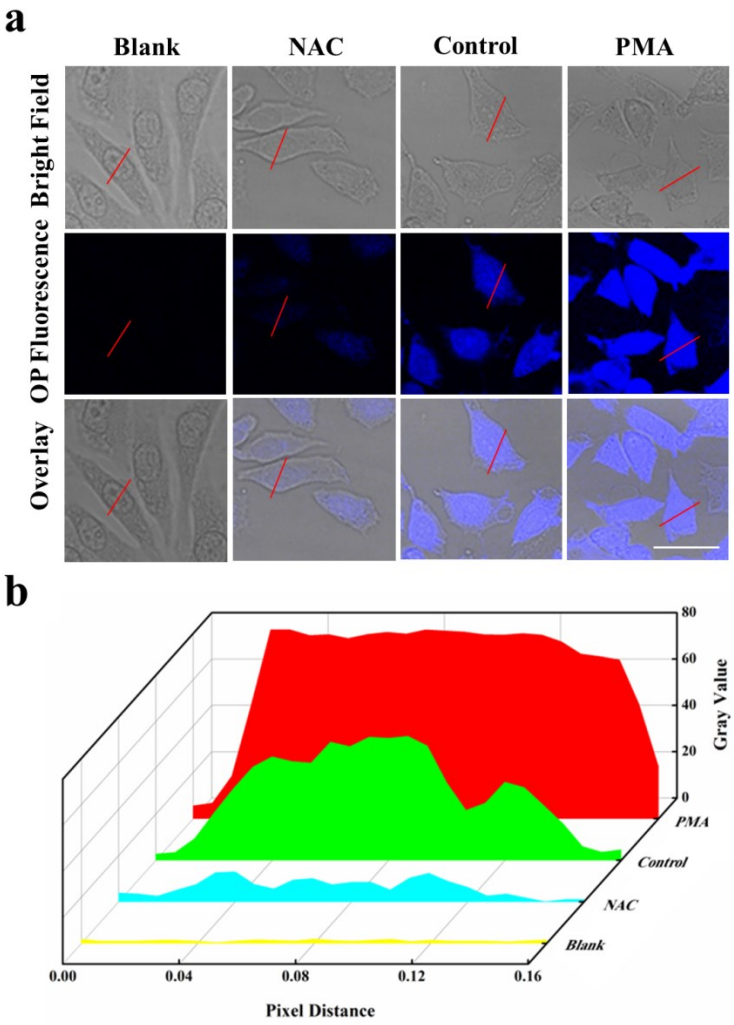

Fig. S19. (a) OP fluorescence imaging of MCF-7 cells were incubated with TP-CQDs@MnO_2 (375.0 μg•mL^{-1}) for endogenous H_2O_2. (b) Grey value of fluorescence intensity of (a). The scale bar is 20.0 μm.

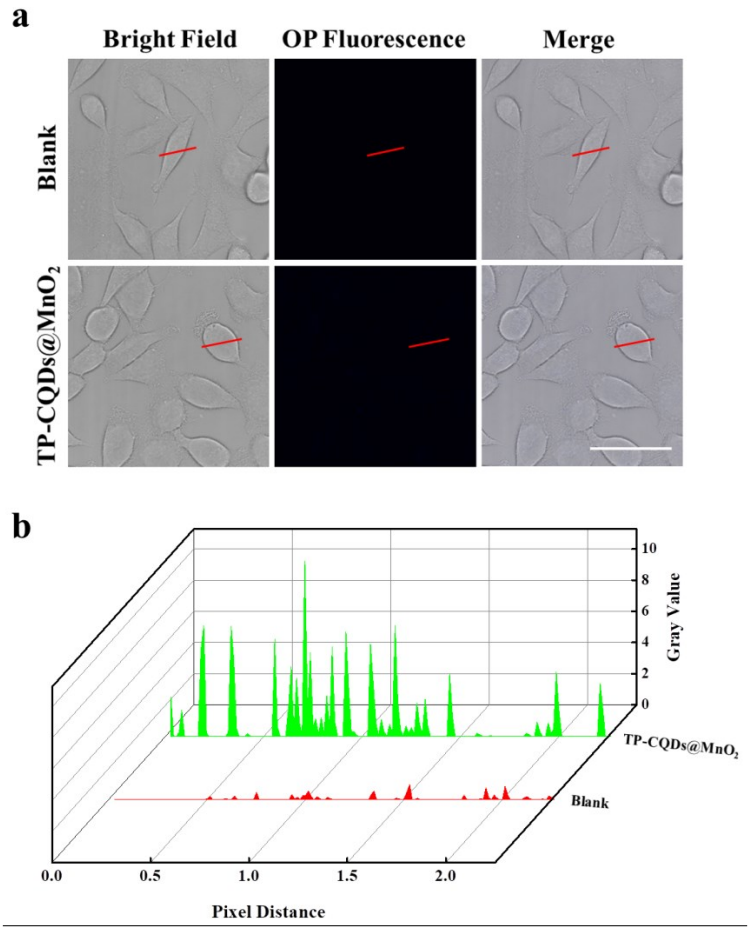

Fig. S20. (a) OP fluorescence images of HEK293T cells were incubated without or with TP-CQDs@MnO_2 (375.0 μg•mL^{-1}) for endogenous H_2O_2. (b) Grey value of fluorescence intensity of (a). The scale bar is 20.0 μm.

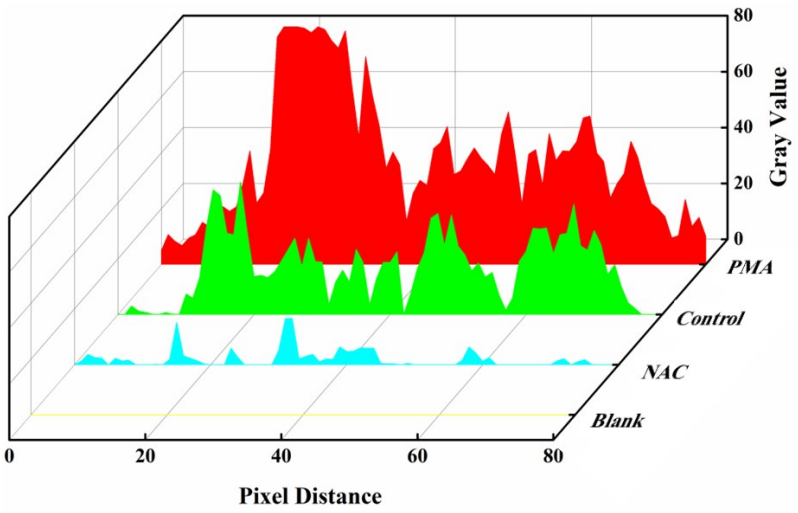

Fig. S21. Grey value of fluorescence intensity of TP fluorescence imaging (Fig. 3, red line) for endogenous H_2O_2 of MCF-7 cells with TP-CQDs@MnO_2 (375.0 μg•mL^{-1}).

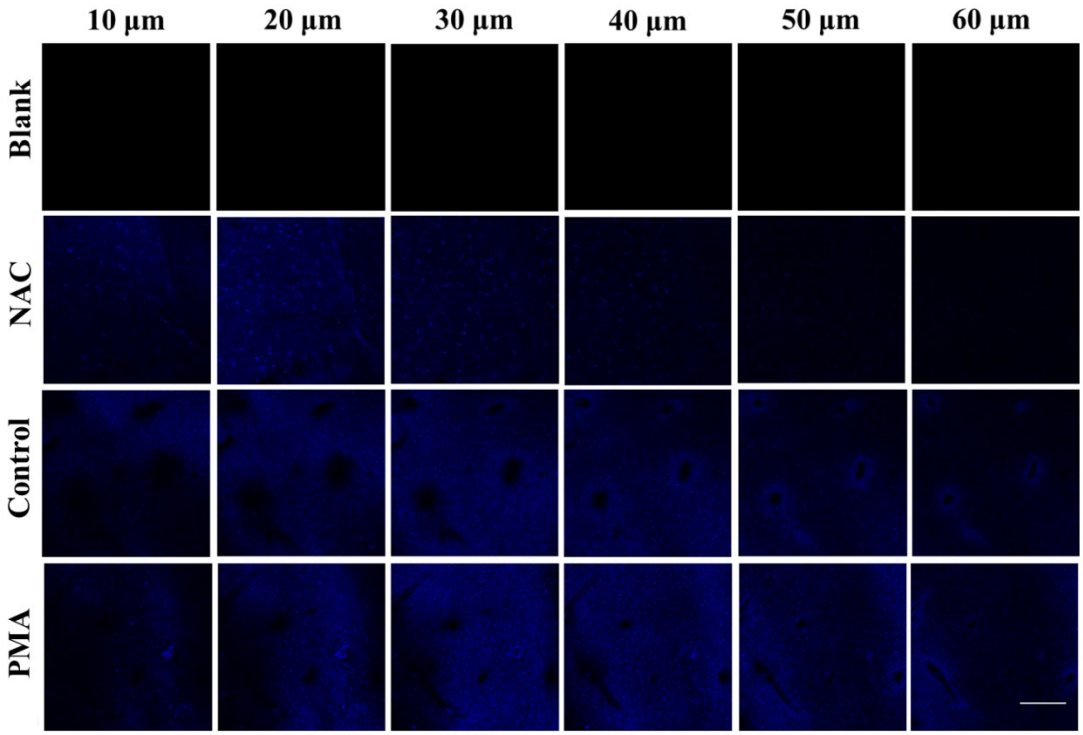

Fig. S22. OP fluorescence imaging of liver tissue slices with TP-CQDs@MnO_2 (375.0 μg•mL^{-1}). The scale bar is 200.0 μm.

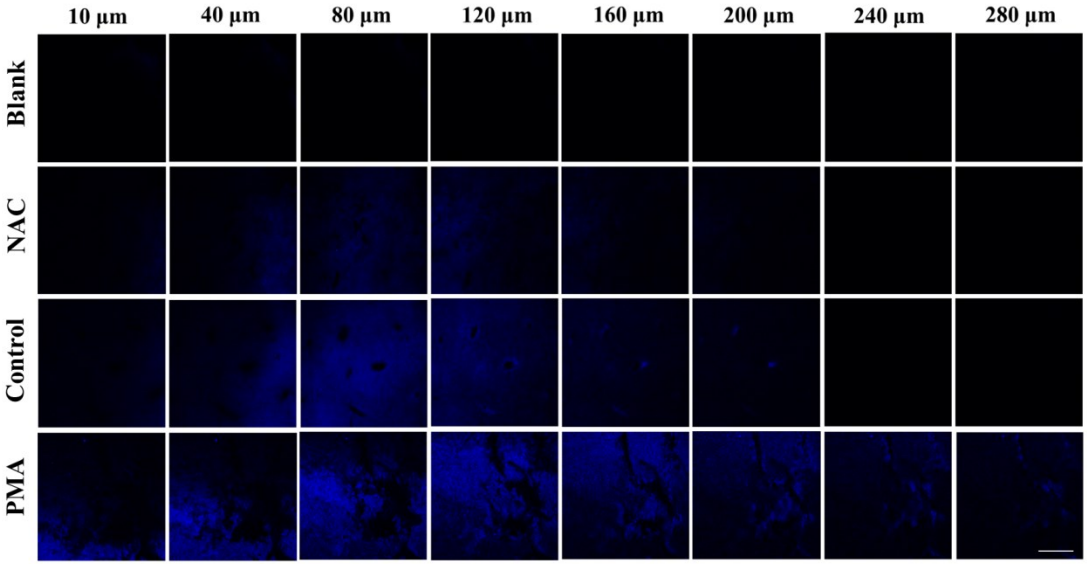

Fig. S23. TP fluorescence imaging of liver tissue slices with TP-CQDs@MnO_2 (375.0 μg•mL^{-1}). The scale bar is 200.0 μm.

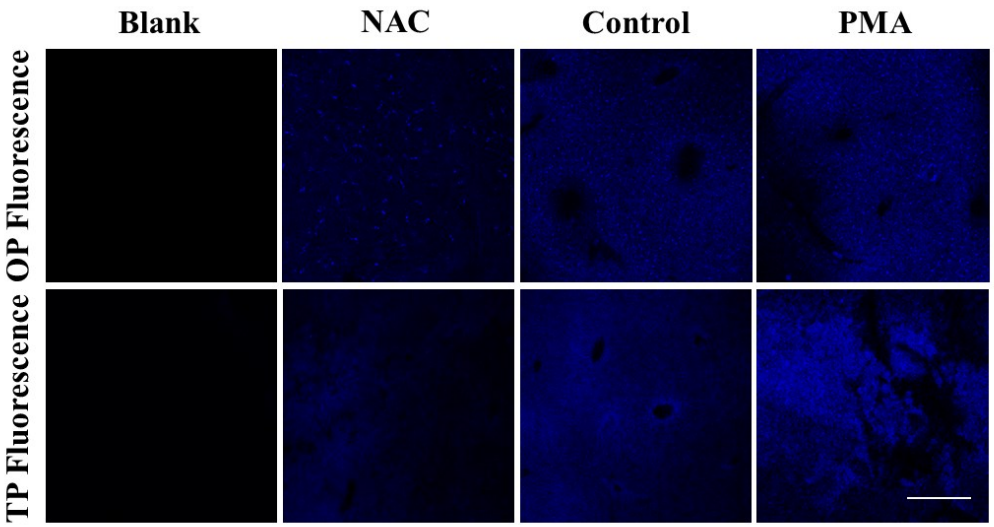

Fig. S24. OP fluorescence (30.0 μm) and TP fluorescence (110.0 μm) imaging of liver tissue slices with TP-CQDs@MnO_2 (375.0 μg•mL^{-1}). The scale bar is 200.0 μm.

18. References

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