Delivery of Cu(II) and Mn(II) by Polydopamine Modified Nanoparticles for Combined Photothermal and Chemotherapy

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Method

Chemicals. β-cyclodextrin (β-CD, >98%, purified by recrystallization for water twice prior to use), urea, dopamine hydrochloride, Triethylenetetramine, 4-methoxybenzenesulfonyl chloride, 3-mercaptopropionic acid, EDC·HCl, NHS, and amantadine were purchased from Aladdin Reagent (Shanghai, China). $NaHCO_3$, NaOH, and $MnCl_2 \cdot 4H_2O$ were purchased from Sheng'ao the chemical reagent Co., Ltd (Tianjing, China). Hexane, methanol, ethanol, N, N-dimethylformamide (DMF), $NH_3 \cdot H_2O$, n-hexane, were purchased from FuYu Chemical (Tianjing, Chain). Cetyltrimethylammonium bromide (CTAB), Tetraethyl orthosilicate (TEOS), triethanolamine (TEOA), anhydrous Na_2CO_3, anhydrous $CuCl_2$ and epichlorohydrin were purchased from Shanghai Macklin Biochemical Co. Ltd.

Characterization. Transmission electron microscopy (TEM) images were obtained with a Hitachi HT7700 operating at 100 kV. The high-resolution TEM images were acquired with Tecnai G2 F20. Scanning electron microscope (SEM) images were obtained with a Hitachi SU8010. Fourier Transform infrared spectrometer (FTIR, Bruker Vertex 70 V) was used to characterize the functional groups of the sample. Using a UV-visible spectrophotometer (UV-8000 S) obtain absorption spectra of the sample. The Brunauer-Emmett-Teller (BET) of N_2 adsorption-desorption curve was determined the specific surface area and pore volume of the sample. ^{1}H NMR spectra were recorded at 400 MHz in NMR solvents and referenced internally to corresponding solvent resonance. Laser Scanning Confocal Microscope images were recorded with Nikon AX R. The apoptosis of cancer cells were recorded by Flow cytometry (ARIA3). Inductively coupled plasma mass spectrometer (ICP-MS, Agilent 8800)

Synthesis of mono-6-deoxy-6-(p-toluenesulfonyl)-β-cyclodextrin (6-OTs-β-CD). The mono-6-deoxy-6-(p-toluenesulfonyl)-β-cyclodextrin (6-OTs-β-CD) was synthesized according to the previous literature[1].

Synthesis of β-CD-NH_2. We prepared the β-CD-NH_2 by referring to the methods mentioned in the literature[2].

Synthesis of ROS-cleavable thioketal linker (TK). The thioketal was synthesized according to the previous literature, 3-mercaptopropionic acid (6 g, 56.53 mmol) and acetone (1.6 g, 27.55 mmol) added into a 50 mL round-bottomed flask. The flask was placed in an ice bath. Then slowly dropped 37% concentrated

hydrochloric acid (4.3 g, 116 mmol) and stirred at room temperature for 12 h. The product was washed with cold n-hexane and three times with cold water. Then TK was dried in a vacuum
at 40 ℃ for 24 h to obtain white powder.

Synthesis of N-((1S,3R,5S)-adamantan-1-yl)-3-((2-((3-(((3s,5s,7s)-adamantan-1-yl)amino)-3-oxopropyl)thio)propan-2-yl)thio)propenamide (Ad-TK-Ad). 1.15 g of TK, 0.55 g of NHS, and 0.95 g of EDC·HCl were added to a 100 mL completely dried reaction flask containing 30 mL of DCM and 4 mL of DMF. Subsequently, 0.6 g of amantadine amine was dissolved in 10 mL of DCM and dropped into the aforementioned mixed solution through a constant pressure funnel. After 48 hours of sealed reaction, the mixture system was first washed three times with saturated sodium chloride solution, and then rinsed once with deionized water. Wash three times with saturated sodium bicarbonate, then wash with deionized water once, and spin steam to obtain a white product.

Synthesis of Poly-CD. Poly-CD was prepared with slight modification according to previous literature[3], 20 g of β-CD dissolved in 30 mL NaOH aqueous solution (15 wt %) by stirring for at least 3 h, and then 0.05 mol EP was added to the mixture. After stirring at 30°C for 3 h, the reaction was stopped by addition of isopropyl alcohol. The precipitate was filtered and dissolved in deionized water, neutralized with HCl, and dialyzed for 7 days (MWCO=3500). The product was obtained by freezing drying, and the yield was 60%.

FITC Labeling of Nanoparticles. 1.0 mL of freshly prepared FITC solution (4.0 mg/mL in DMSO) was added into a dispersion of MNZ (10 mL, 1.0 mg/mL), and the mixtures were stirred for 24 h in the dark. Then the FITC-labeled MNZ was obtained by centrifugation (15000×g, 10 min) and washing with deionized water for further use.

Detection of $^1O2$. 100 ug/mL H-MnO_2, 100 μM H_2O_2 and 25 ug/mL DPBF were incubated in 0.1 M Hac-NaAc buffer (pH 6.0) for 0-400 s. Then using a UV-vis spectrometer to record the absorbance changes of DPBF.

Detection of ·OH in vitro. 2 mL of H_2O_2 (30 mmol/L), 2 mL of methylene blue (15 ug/mL) and 2 ml of various concentrations of H-MnO_2 (0-200 ug/mL) were mixed for 10 minutes. Then using a UV-vis spectrometer to record the absorbance changes of methylene blue.

Measurement of peroxidase-like activity and steady-state kinetic assays. The assays were implemented according to the literature with some modification. The assay was performed by adding different concentration of H-MnO_2 (0 ug/mL, 18.75 ug/mL, 37.5 ug/mL, 75 ug/mL, 112.5 ug/mL, and 150 ug/mL). Different concentration catalysts were added into 0.01 M HAc-NaAc buffer (pH=4.0, pH=5.0, pH=6.0, and pH=7.4), after that 1 mM TMB was added and the mixture solution was

incubated for 5 minutes. Then using a UV-vis spectrometer to record the absorbance changes.

Electron paramagnetic resonance (ESR) detect the reactive oxygen species. For the ESR assay, DMPO was used as the ·OH trapping agent. In a typical experiment, 100 μg/mL H-MnO_2 was added into the weak acidic buffer (0.01 M HAc-NaAc buffer, pH 6.0) containing 1.0 mM H_2O_2 and 100 μM DMPO. After violent ultrasonication for 60 s, the mixture was transferred to a quartz tube for ESR assay.

Degradation of H_2O_2 In Vitro. The prepared H-MnO_2@PDA and H-MnO_2 were added into H_2O_2 (1 mM) solution and reacted for 30 min. After centrifugation, the supernatant (100 μL) of these samples were mixed with H_2O_2 detection Kit (100 μL) for 30 min. Then, the absorption peak at 415 nm was measured.

Degradation of GSH In Vitro. The prepared H-MnO_2@PDA and MNF were added into GSH solution (10 mM) and reacted for 30 min. After centrifugation, the supernatant (100 μL) of these samples were mixed with 5,5′-dithiobium (2-nitrobenzoic acid) (DTNB, 250 μL, 2.5 mg mL^{-1}) for 30 min. Then, the absorption peak at 410 nm was measured.

Generation of O_2 In Vitro. H-MnO_2 were added into H_2O_2 (1 mM) and reacted for different pH conditions, respectively. The generation of O_2 was recorded by photos.

The Release Curve of Mn and Cu. The prepared MNF nanoparticles was mixed with PBS, GSH (10 mM), H_2O_2(1 mM), and GSH (10 mM) + H_2O_2 (1 mM) for 30 min. Then, the released Mn and Cu at different time was recorded by ICP-MS.

Detection of superoxide dismutase-like activity. The SOD-like activity of H-MnO_2@PDA was determined by SOD assay kit according to the requirements of the manufacturer. H-MnO_2@PDA solutions with different concentrations ranged from 0 to 150 μg/mL**.** The mixture was incubated at 37℃ in the dark, and finally its absorbance was measured.

Calculation of the photothermal conversion efficiency. The photothermal conversion efficiency of MNF was measured following Roper's method. MNF (1.0 mL 400 μg/mL) was added in a glass vial and irradiated by 808 nm laser (1.5 W cm^{-2}) for 600 s, then turn off the laser until the solution cooled to room temperature. The temperature of MNF was monitored by a thermocouple microprobe submerged in the solution. The experiment was repeated four cycles to investigate the photothermal stability of the material.

Cell culture. HeLa cells were incubated in MEM containing 10% FBS and 1% antibiotics at 37℃ in a humidified atmosphere containing 5% CO_2. L929 cells were

incubated in 1640 containing 10% FBS and 1% antibiotics at 37℃ in a humidified atmosphere containing 5% CO_2.

Cellular uptake. HeLa cells were seeded in confocal dishes at a density of 2*10^5 cells per dish and cultured for 24 h. After replacing the medium with 1 mL of the DOX@MNZ, cells were further incubated for different time (0 h, 1 h, 2 h, 4 h, 8h). Then, the medium was replaced by 1.50 mL DMEM containing 10 μL Hoechst 33342 (0.4 mg/mL). After 15 minutes of incubation, the medium was removed and the cells were washed three times with PBS. The fluorescence imaging of cells was recorded by confocal microscopy.

Detection of ROS in cells. ROS generation in cells was measured by using an ROS Assay Kit. HeLa cells were seeded in 6-well plates at a density of 2×10^5 cells per well and cultured with acidified MEM supplemented with 10% FBS. After incubation at 37°C in 5% CO_2 for 24 h, the cells were treated with 1.0 mL 200 μg/mL MNZ (with or without 100 μM H_2O_2 and NIR) and incubated for another 12 h. Then, the medium was removed and the MEM containing DCFH-DA (10 μM) and 10 μL Hoechst 33342 (0.4 mg/mL) was added. After 15 minutes of incubation, the medium was removed and the cells were washed three times with PBS. The fluorescence imaging of cells was analyzed by CLSM.

In vitro cytotoxicity and biocompatibility study. The biocompatibility of NPs was investigated using the CCK-8 assay. L929 cells a were seeded in the 96-well plates at a density of 6,000 cells per well for 24 hours. The cells were co-incubated with MNZ at different concentrations for 48 hours. After incubation, CCK-8 was added to each well and incubated for another 1 hours. Then, the absorbance measured at 450 nm. HeLa cells were seeded in 96-well plates at 4000 cells per well and cultured with 100 μL of neutral or acidified MEM containing 10% FBS for 12 h. The cells were treated with MNZ (with or without 100 μM H_2O_2) with different concentrations at weak acid condition, and further divided into two groups, one group was irradiated and the other were not. After incubating for four hours to ensure that the material was internalized by cells, the irradiation grouped was exposure to 808 nm laser with 1.5 W/cm^2 for 5 minutes. After further incubation for 20 h, the medium was replaced with fresh neutral culture medium and CCK-8 solution was added. The cells were incubated for another 1 h. Then, the absorbance measured at 450 nm.

For live/dead cell staining assay. HeLa cells were seeded in 6-well plates at 2×10^5 cells per well in 2 mL of neutral DMEM containing 10% FBS and incubated for 24 h under dark condition before treatment. After that, the MEM was replaced with fresh MEM containing H-MnO_2 and MNZ for 12 h incubation. The cells were washed for three times with PBS, followed by staining with FDA (10 μM) and PI (20 μM). After 20 minutes of incubation, the medium was removed and the cells were washed three times with PBS. The fluorescence imaging of cells was analyzed by CLSM.

For analysis of cell apoptosis. Annexin V-FITC and PI assay was employed. HeLa cells were seeded in 6-well plates at a density of 2*10^5 cells per well and incubated 24 h. Subsequently, the MEM was replaced with fresh acidified MEM containing H_2O_2 (100 μM), H-MnO_2 (200 μg/mL), MNZ (200 μg/mL), MNZ (200 μg/mL) plus H_2O_2 (100 μM).

Evaluation of GSH in cell. The HeLa cells were seeded and incubated for 24 h. Then the cell incubated with different concentration of nanoparticles (0, 25, 50, 75, and 100 μg/mL) for 12 h. After that, the GSH in cell was tested by a Reduced Glutathione (GSH) Colorimetric Assay Kit.

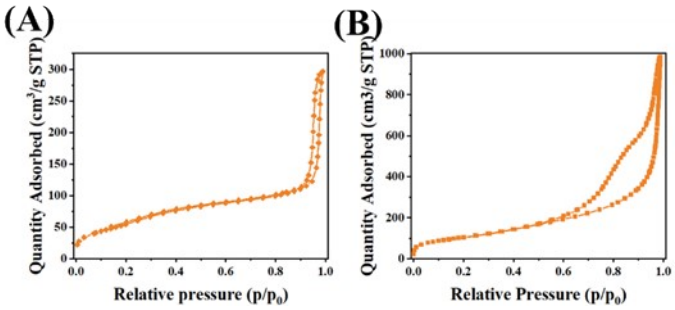


Figure 1S. N_2 absorption-desorption curves of (A) SiO_2 and (B) H-MnO_2.

Figure 2S. (A) Digital photographs of oxidase-like activity of H-MnO_2, the effect of pH and concentrations on the oxidase-like activity. (B) Imaging for peroxidase-like activity of H-MnO_2, the effect of pH and concentrations on the peroxidase-like activity.

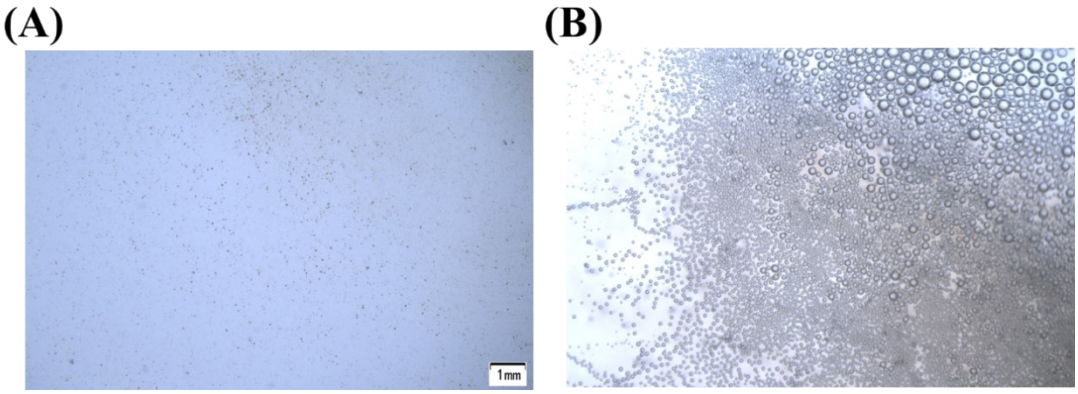


Figure 3S. Stereomicroscopic imaging of the generation of O_2 (A) H-MnO_2 and (B) H-MnO_2 + H_2O_2.

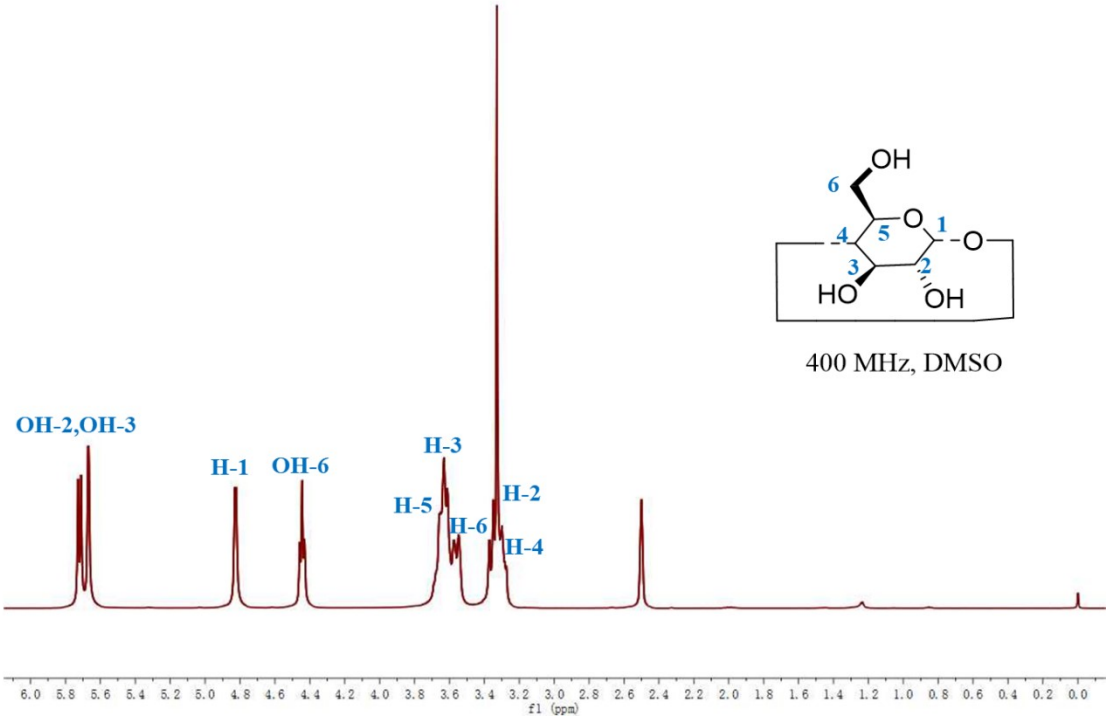


Figure 4S. ^{1}H NMR spectrum (400 MHz, DMSO) of β-cyclodextrin (β-CD).

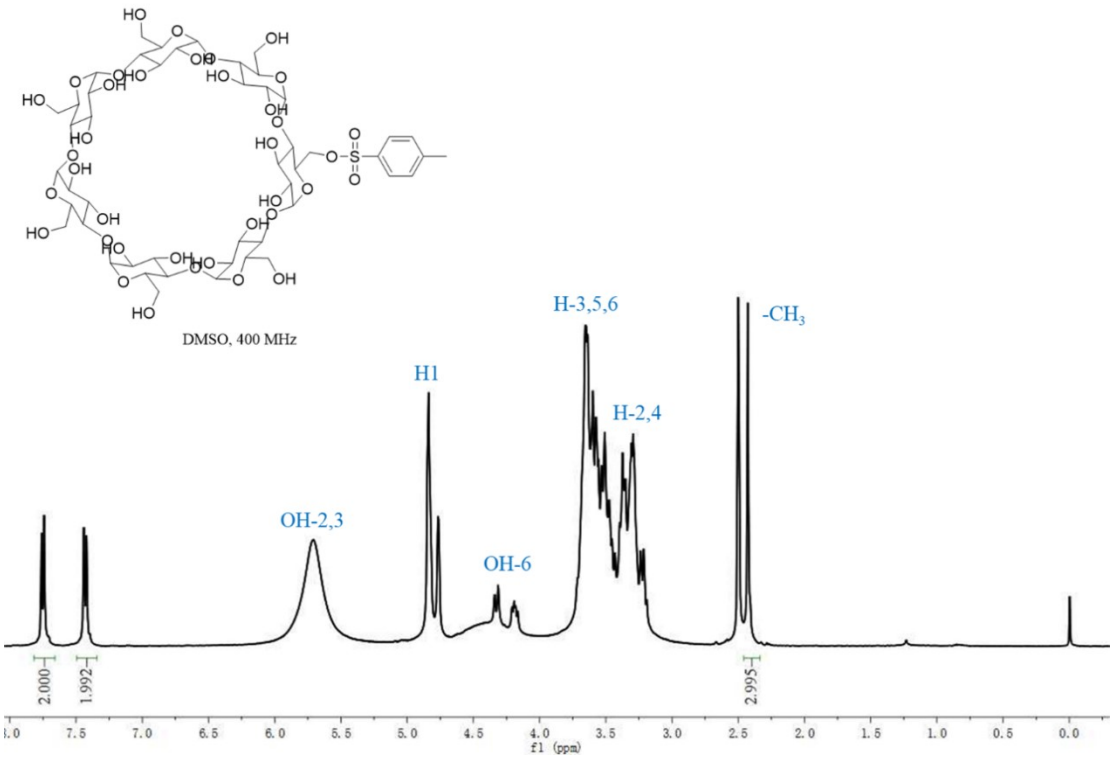


Figure 5S. ^{1}H NMR spectrum (400 MHz, DMSO) of β-CD-OTs.

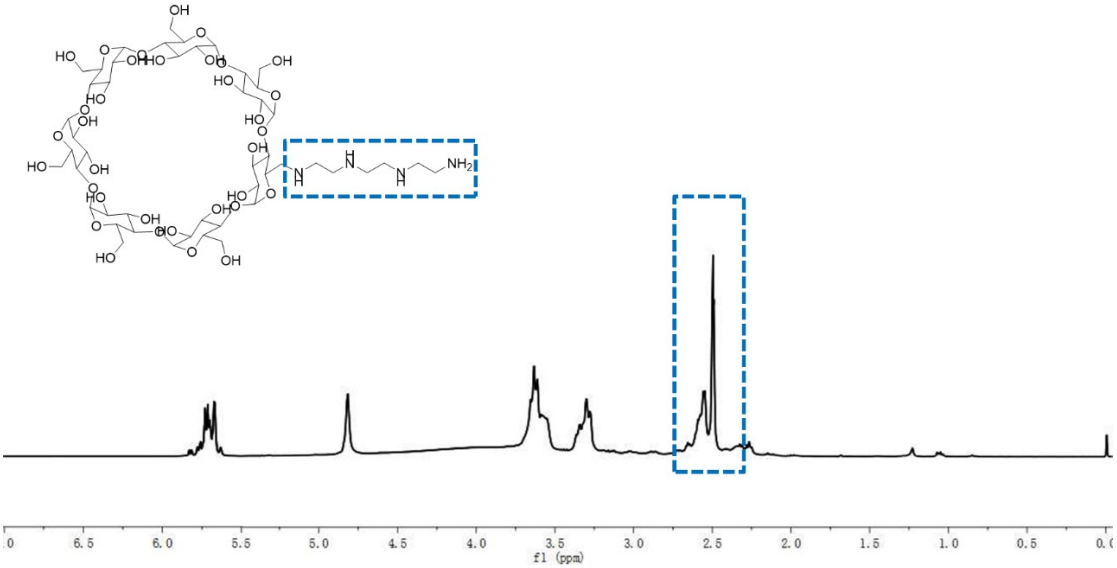


Figure 6S. ^{1}H NMR spectrum (400 MHz, DMSO) of β-CD-NH_2.

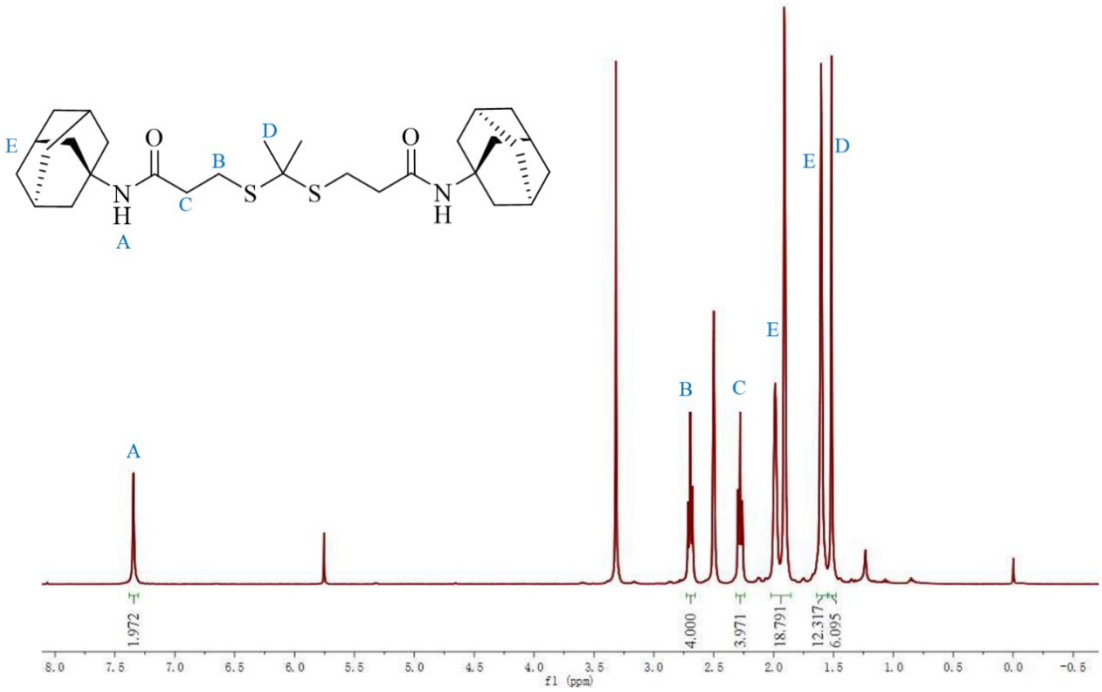


Figure 7S. ^{1}H NMR spectrum (400 MHz, DMSO) of Ad-TK-Ad.

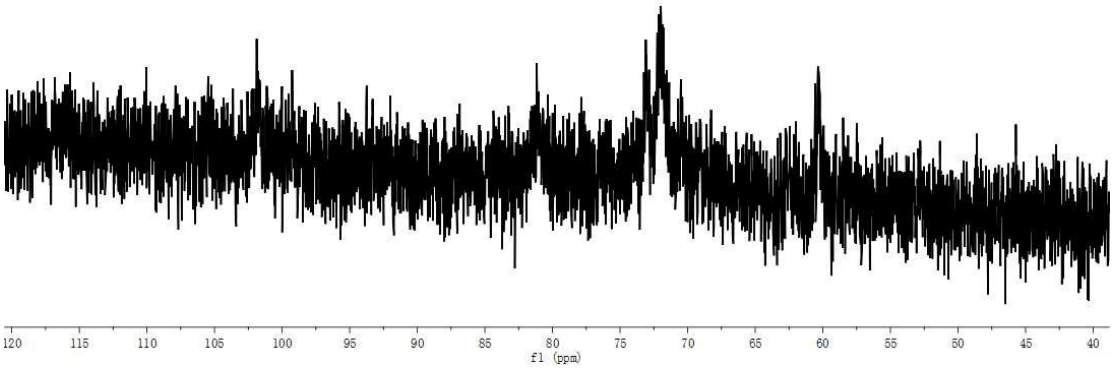


Figure 8S. The ^{13}C NMR (DMSO, 400 MHz) spectrum of poly-cyclodextrin (Poly-CD).

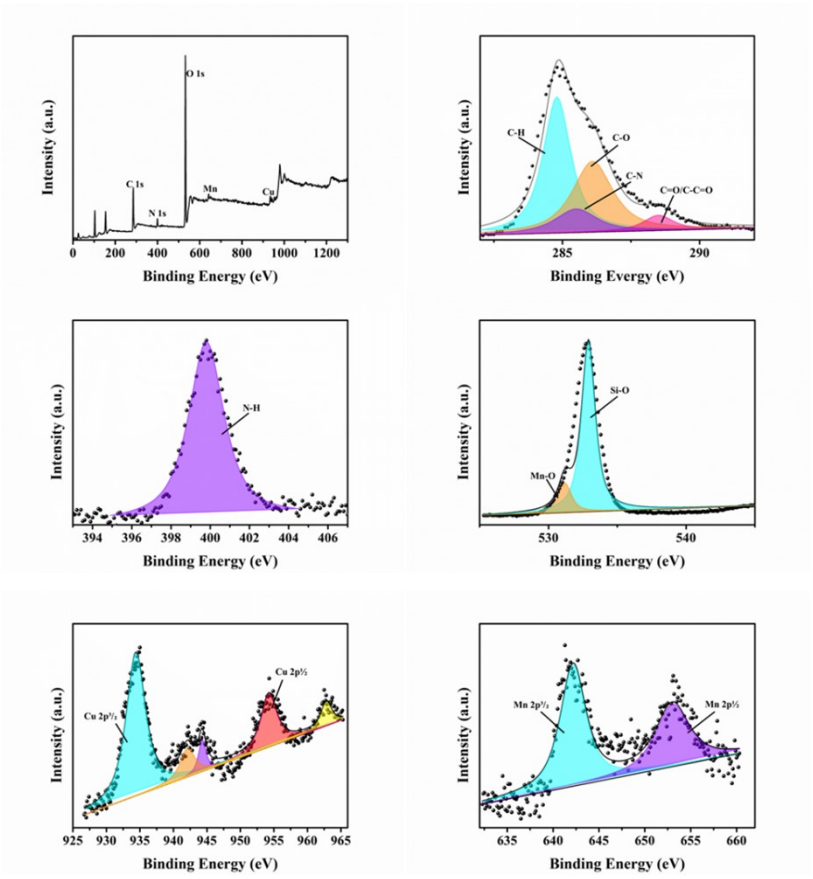
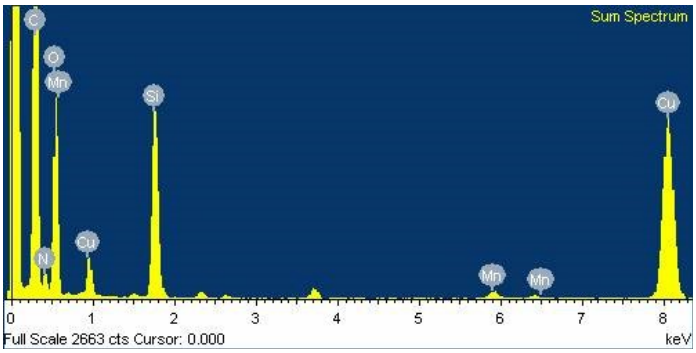


Figure 9S. XPS spectrum of MNS.



Element	Weight%	Atomic%
C K	46.50	68.06
N K	1.24	1.55
O K	15.10	16.60
Si K	9.96	6.23
Mn K	0.66	0.21
Cu K	26.54	7.34
Totals	100.00	

Figure 10S. The element content of MNZ.

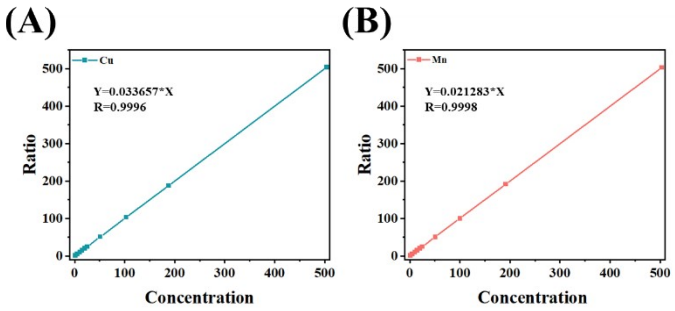


Figure 11S. The standard curves of (A) Cu and (B) Mn.

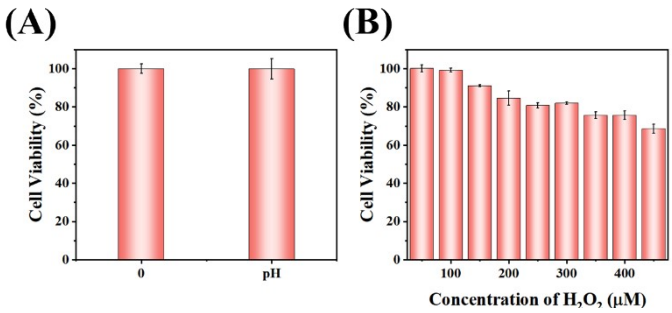


Figure 12S. (A) The effect of pH on the viability of HeLa cells. (B) The effect of H_2O_2 on the viability of HeLa cells.

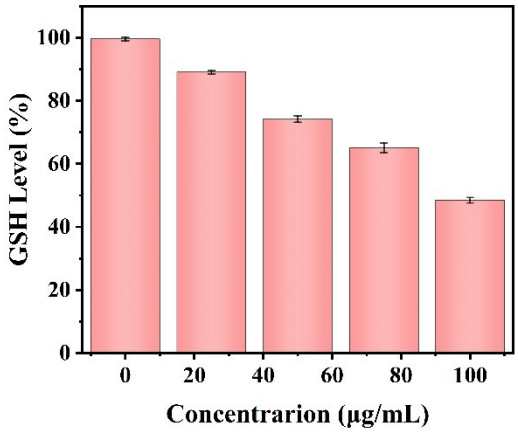


Figure 13S. Intracellular GSH depletion with different concentrations of MNZ.

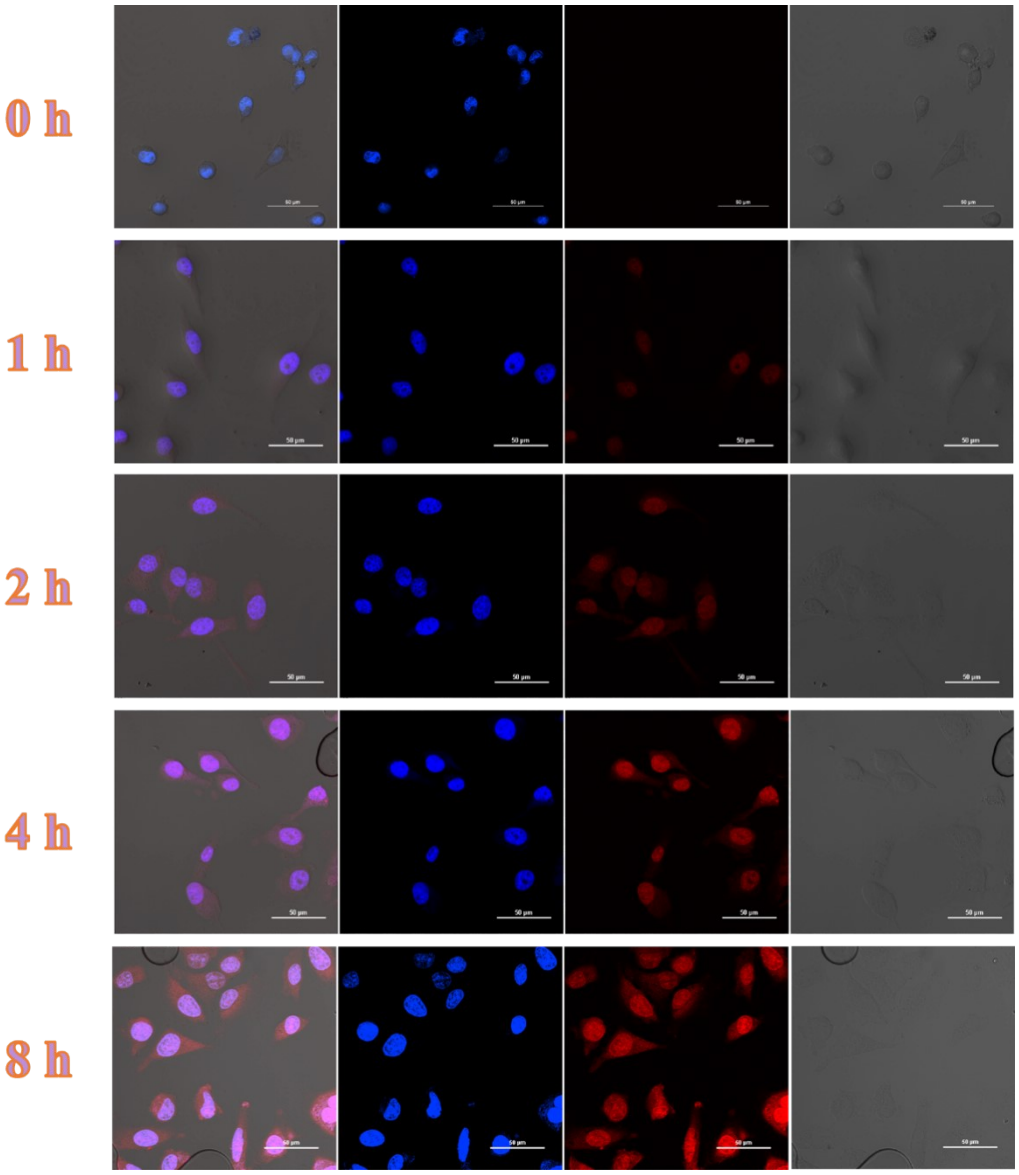


Figure 14S. (A) CLSM images of Hela cells treated with DOX@MNZ at different times. Blue represented Hoechst 33342 and red represented DOX.

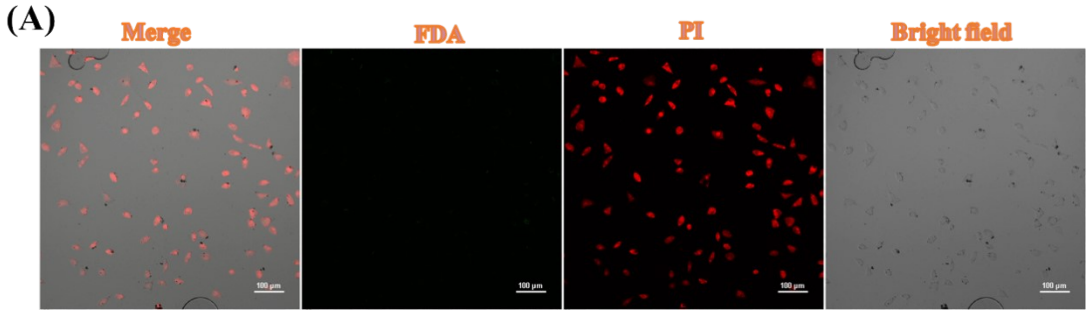


Figure 15S. Live/dead cell assay. Cellular response to the treatment of MNZ in cells. Hela cells were treated with nanoparticles, NIR and H_2O_2 (100 μM) at pH 6.8. Scale bars = 100 μm.

Reference

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3. J. Liu, W. E. Hennink, M. J. van Steenbergen, R. Zhuo and X. Jiang, *Bioconjugate Chemistry*, 2016, **27**, 1143-1152.