Supplementary Information

Pt Nanozyme for O$_2$ Self-Sufficient, Tumor Specific Oxidative Damage and Drug Resistance Reversal

Zhaoyu Ma,a Long Wu,b Kai Han,*b and Heyou Han*ab

a. State Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, Hubei, P. R. China Address here.
b. State Key Laboratory of Agricultural Microbiology, College of Science, Huazhong Agricultural University, Wuhan 430070, Hubei, P. R. China

Email: hyhan@mail.hzau.edu.cn
hank@mail.hzau.edu.cn

Experimental Section

Materials: Tetraethyl orthosilicate (TEOS, 99%), (3-mercaptopropyl) trimethoxysilane (MPTS, 95%), 3-aminopropyl trimethoxysilane (APTMS, 97%), H$_2$O$_2$, and all other reagents were of analytical reagent grade and used as received. calcein-AM, PI, Hoechst 33342 and 5-Carboxyfluorescein (FAM) were provided from Sigma-Aldrich (USA), and Qiancheng Technology Co. Dulbecco’s modified Eagle’s medium (DMEM), Roswell Park Memorial Institute (1640), trypsin, fetal bovine serum (FBS) and MTT, penicillin-streptomycin were purchased from GIBCO Invitrogen Corp. Ultrapure water (18.2 MΩ; Millpore Co., USA) was used in all experiments and to prepare materials.

Synthesis of Au@SiO$_2$ and PtAu@SiO$_2$: SiO$_2$ nanospheres were synthesized using a seed growth method (Stöber). Then 40 mL of HAuCl$_4$ (0.25 mM) and sodium citrate (0.25 mM) were taken into an ice-water bath, and then fresh NaBH$_4$ solution was added under constant stirring. After the solution was changed from bright yellow to burgundy, it was left for 15 minutes to get Au NPs. To get Au@SiO$_2$ Nanoparticles, 2 mL of the above-prepared aminated SiO$_2$ nanospheres solution was added to a 50 mL glass vial, and 15 mL Au NPs solution was added under stirring condition. 30 minutes later, the solution was centrifuged and washed three times to remove excess Au NPs and the purified Au@SiO$_2$ nanoparticles were dispersed in water. The above-prepared Au@SiO$_2$ were added to a flask...
and stirred at room temperature. Subsequently, H₂PtCl₆ (0.2 mL, 19.3 mM) and sodium citrate (1 mL, 1%) were added and stirred for 15 minutes so that H₂PtCl₆ was fully adsorbed on the surface of the Au@SiO₂. Finally, fresh NaBH₄ solution was added and the reaction was stirred for a further 15 minutes. The solution was centrifuged and washed for three times to obtain PtAu@SiO₂.

**Characterization of PtAu@SiO₂:** Morphology of PtAu@SiO₂ was observed via TEM (JEM-2100 microscope). The hydrodynamic size and zeta potential of PtAu@SiO₂ in deionized water were measured by a Nano-ZS3600 (Malvern Instruments) at 25°C. Energy-dispersive X-ray EDS mapping images (Si, Au and Pt) were analyzed by Four-Detector designed Super X Energy Dispersive Spectrometry. X-ray photoelectron spectroscopy (XPS) spectra were analyzed by Thermo Fisher Scientific ESCALAB 250Xi Spectrometer Electron Spectroscopy (America).

**pH Responsive Release of Platinum Ion of PtAu@SiO₂:** 0.5 mL PtAu@SiO₂ solution was positioned in a dialysis bag (MWCO 500 Da) and dialyzed in 10 mL of PBS buffer with different pHs of 6.5 and 7.4. The dialysate was collected at preset times and fresh PBS buffer was added. The leached platinum ion of different dialysate was determined via ICP-MS.

**Oxidase Mimetic PtAu@SiO₂:** The TMB oxidation by PtAu@SiO₂ was conducted in the absence of H₂O₂. 50 µL TMB were respectively added into the same amount of PtAu@SiO₂ (20 mg/L, 100 µL) and Au@SiO₂ solution (PBS buffer, pH 6.5 or 7.4). After 5 minutes, the photos were obtained. In order to verify the color change sensitivity of TMB in different pHs, PtAu@SiO₂ was incubated with TMB in PBS buffer with different pHs of 4, 5, 6, 7 and 8. Three PtAu@SiO₂ solution samples were placed in pH 6.5 PBS buffer, one was purged with nitrogen, one was purged with oxygen and the other placed in air. After 20 minutes, samples (100 µL) were added to each tubes containing 100 µL TMB. The color change was recorded.

**PtAu@SiO₂ Mediated H₂O₂ Decomposition and Oxygen Production:** Various concentrations of PtAu@SiO₂ (0.2, 1, 2 mg/L) were incubated with H₂O₂ (10 mM), respectively. The oxygen evolution over time of the above samples was recorded through a portable dissolved oxygen meter (Shanghai Ray magnetic JPB-607A). Besides, the oxygen generation of PtAu@SiO₂ incubated with different concentrations of H₂O₂ (0, 1, 10, 50 mM) for 10 min was also observed.

**PtAu@SiO₂ Mediated ROS Generation:** The generation of ROS was measured via fluorescence spectroscopy using DCFH-DA as the sensor. 8 mL of 0.01 M NaOH was mixed with 2 mL of DCFH-DA in methanol (0.5 mg/mL) in the dark for 30 min at room temperature to chemically hydrolyze
DCFH-DA to DCFH. Then, 40 mL of the PBS buffer (10 mM, pH 7.4) was added to stop the reaction. The stock solution of DCFH was kept in a -20°C in the dark before use. For fluorescence spectrum test, collected PtAu@SiO$_2$ and equal amount Au@SiO$_2$ was redispersed in PBS buffer with different pHs (6.5, 7.4 and 6.5+Vc, 10 mM, 10 mg/L), then 100 µL of NaOH pretreated DCFH-DA was added. Then the fluorescence spectroscopy of the mixtures at a preset time was recorded via fluorescence spectrophotometer (Ex: 485 nm; Em: 495~600 nm). To distinguish the type of ROS, the electron spin resonance (ESR) technique was employed to monitor ROS signals from PtAu@SiO$_2$ using BMPO and DMPO as the spin-trapping agents for $\cdot$O$_2^{-}$ and $\cdot$OH, respectively. Spectra of spin trapped $\cdot$O$_2^{-}$ and $\cdot$OH were acquired by mixing 5 µL BMPO (500 mM) or DMPO (5 µL, 500 mM) with 100 µL PtAu@SiO$_2$ or Au@SiO$_2$ in different pHs of 6.5 and 7.4.

**Intracellular ROS Production:** HeLa cells were seeded into culture dishes in 5% CO$_2$ at 37°C for 24 h. Then, Fresh H$_2$DCF-DA (10 mM) was added into each well and the cells were incubated in 5% CO$_2$ at 37°C for 20 min. After the cells were washed with PBS for three times, the cells were treated with PtAu@SiO$_2$, Au@SiO$_2$ in different pH medium (pH 6.5, 7.4) and PtAu@SiO$_2$ + Vc (4 µL, 1 mg/mL) in pH 6.5 medium. Subsequently, the cells were washed with PBS and then were immediately captured by the confocal laser scanning microscopy (CLSM).

**Cytotoxicity Assays:** HeLa Cells and A549/DDP were seeded in 96-well plates in 5% CO$_2$ at 37°C for 24 h, respectively. Then the cells were treated with PtAu@SiO$_2$, Au@SiO$_2$ in different pHs of 6.5 and 7.4 and PtAu@SiO$_2$ in the presence of Vc (8µL, 1 mg/mL) in pH 6.5, respectively. After 40 h incubation, the medium was replaced with fresh medium containing 30 µL MTT (5 mg/mL) each well. The supernatant was replaced with 150 µL DMSO to dissolve the formazane at post of 4 h incubation. The optical density (OD) values at 490 nm were recorded by a microplate reader (Bio-Rad, Model 550, USA). The relative cell viability was calculated by the following formula: cell viability (%) = OD$_{(sample)}$/OD$_{(control)}$ × 100%. OD$_{(sample)}$ was the OD value in the presence of sample and OD$_{(control)}$ was the optical density in the absence of sample.

**Calcein AM/PI Assay:** HeLa Cells and A549/DDP were seeded 35 mm diameter dishes in 5% CO$_2$ at 37°C for 24 h, respectively. Then the cells were treated with PtAu@SiO$_2$, Au@SiO$_2$ in different pHs of 6.5 and 7.4 and PtAu@SiO$_2$ in the presence of Vc (8 µL, 1 mg/mL) in pH 6.5, respectively. Cells were incubated for 40 h. The cells were detected observed using CLSM after co-staining with calcein AM (2 µM) and PI (3 µg/mL) for 20 min.
Cytotoxicity in Different $O_2$ Concentrations: For studying the remission of hypoxic environment of PtAu@SiO$_2$, the A549/DDP cells were seeded in 96-well plates and 35 mm diameter dishes in normoxic (21% $O_2$, 5% $CO_2$, 74% $N_2$) or hypoxic (5% $O_2$, 5% $CO_2$, 90% $N_2$) condition for 24 h. After that, PtAu@SiO$_2$ and cisplatin, at the indicated concentrations, were added to the cell culture medium and incubated with cells for 40 h. Finally, the ability to alleviate hypoxia was judged by MTT assay and observed via CLSM after co-staining with calcein AM (2 µM) and PI (3 µg/mL) for 20 min.

Investigating the uptake mechanism: In order to track the entry of PtAu@SiO$_2$ into cells in real time, fluorescent dyes (5-Carboxyfluorescein, FAM) were attached to PtAu@SiO$_2$ (named PtAu@SiO$_2$-FAM) through a reaction between the amino group on the surface of the SiO$_2$ and the carboxyl group of the fluorescein dyes. A549 cells were pre-incubated in DMEM for 1 h at 37 °C with different pathway inhibitors such as chlorpromazine (10 mg/L, clathrin-mediated endocytosis inhibitor), genistein (50 mg/L, caveolae-mediated endocytosis inhibitor), and amiloride (4 mg/L, macropinocytosis inhibitor). Moreover, A549 cells were pre-cultured at 4 °C for 1 h to investigate the influence of energy on the cell internalization process. After that, the cells were washed with PBS, followed by incubation with PtAu@SiO$_2$-FAM for 4 hours at 37 °C. The mean fluorescence intensity of cells treated with various aforementioned inhibitors was compared with the control group (treated without any cellular uptake inhibitors) by flow cytometry. To obtain the ratios of cellular uptake, the cells treated as above than were incubated with fresh aqua regia overnight and then diluted with water for ICP-MS analysis. For cell colocalization, after different inhibitors and subsequent PtAu@SiO$_2$ treatment, the cells were washed with PBS in order to remove PtAu@SiO$_2$ that was not absorbed. Subsequently, the cells were co-cultured with 1640 containing H33342 dye (10 mg/L) for 30 minutes. Finally, the cells were washed with PBS and then were immediately captured by the confocal laser scanning microscopy (CLSM), nucleus (blue, Ex:405 nm, Em:440~480 nm); PtAu@SiO$_2$-FAM (green, Ex:488 nm, Em:495~550 nm).

Cell Cycle progression by Flow Cytometry: A549/DDP cells (in the exponential growth phase) were seeded in 6-well plates in normoxic (21% $O_2$, 5% $CO_2$, 74% $N_2$) or hypoxic (5% $O_2$, 5% $CO_2$, 90% $N_2$) condition for 24 h. After that, the cell culture medium was replaced by fresh culture medium (pH 6.5) with or without PtAu@SiO$_2$. Then, the cells were repositioned in a hypoxic environment for 24 hours. Similarly, the cells under normoxia were treated with PtAu@SiO$_2$-containing medium (pH 6.5)
and cultured under normoxia for 24 hours. After PtAu@SiO2 treatment in different environments, the cells were washed, collected, re-suspended in ice-cold PBS and fixed using 70% ethanol at 4°C for over 24h. Thereafter, cells were centrifuged, re-suspended in ice cold PBS and then stained with PI using the cell cycle and apoptosis analysis kit (Beyotime Biotechnology, China) at 37°C in the dark for 30 minutes. Cells were then detected using the BD FACSCalibur CellSorting System and results were analyzed using FlowJo 7.6.1 software.

Gene Analysis: A549/DDP cells (in the exponential growth phase) were seeded in 6-well plates in hypoxic (5% O2, 5% CO2, 90% N2) condition for 24 h. After that, the cell culture medium was replaced by fresh culture medium (pH 6.5) with or without PtAu@SiO2. Then, the cells were repositioned in a hypoxic environment for 24 hours. After that, the cells were washed three times and send to Shanghai Majorbio Bio-pharm Technology Co., Ltd for eukaryotic transcriptome sequencing.
**Figure S1.** Schematic showing the estimation of Pt shell thickness (x nm) for the PtAu NPs on the PtAu@SiO₂ shown in Figure 1.
Figure S2. Fluorescence spectra of DCF by adding a) Au@SiO$_2$ over time at pH 6.5 and b) PtAu@SiO$_2$ over time at pH 6.5 in the presence of Vc.
**Figure S3.** The potential process of PtAu@SiO$_2$ catalyzed generation of superoxide anion and hydroxyl radical in a hypoxic tumor microenvironment. PtAu@SiO$_2$ nanozyme firstly catalyze H$_2$O$_2$ into O$_2$ and then catalyze O$_2$ to generate superoxide anion and hydroxyl radical under hypoxic conditions.
Figure S4. Cell viability of PtAu@SiO₂ against COS7 cells at pH 7.4.
Figure S5. The production of ROS in A549/DDP cells treated with PtAu@SiO$_2$ under different environments (pH 7.4, pH 6.5 and pH 6.5+Vc). DCF fluorescence (green) depicted intracellular ROS. The scale bar was 50 µm.
Figure S6. Digital photos of PtAu@SiO$_2$ producing oxygen at pH 6.5 a) after reaction with increasing concentrations of H$_2$O$_2$: 0 mM, 1 mM, 10 mM, 50 mM; PtAu@SiO$_2$: 3 mg/L; and b) after reaction with increasing concentrations of PtAu@SiO$_2$: 1 mg/L, 5 mg/L, 10 mg/L; H$_2$O$_2$: 10 mM.
**Figure S7.** Quantitative analysis of light intensities of a) P53 and b) P21 protein expression, as the ratio of protein to GAPDH from Western blot results.
References