Supporting Information

Mitochondrial Reactive Oxygen Species Burst for Cancer Therapy

Triggered by Near-Infrared Light

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Keywords: near-infrared spectroscopy, reactive oxygen species, mesoporous Pt shell, core-shell nanoparticles, PDT/PTT
1. Materials

Chloroauric acid tetrahydrate (HAuCl₄·4H₂O), potassium tetrachloroplatinate(II) (K₂PtCl₄), trisodium citrate dihydrate (Na₃C₆H₅O₇), ascorbic acid (AA), Brij 58, 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), (4-carboxybutyl) triphenylphosphonium bromide, ethylisopropylamiloride (EIPA), chlorpromazine, dynasore, filipin, dimalonic acid (ABDA), singlet oxygen sensor green and 2,7-dichlorodihydrofluoresceindiacetate (DCFH-DA) were purchased from Sigma-Aldrich (MA, USA). Amino Poly(ethylene glycol) amine (2000 Da) and Folic acid Poly(ethylene glycol) amine (2000 Da) were purchased from NANOCS (NY, USA). Chlorin e6 (Ce6) was obtained from the Department of Integrative Physiology and Neuroscience, Washington State University (WSU). Hydrochloric acid (HCl, 39%) and sodium hydroxide (NaOH) were of analytical grade and purchased from local suppliers. Fetal bovine serum (FBS) and bovine serum albumin (BSA) were purchased from ATCC. Dulbecco's Modified Eagle's Medium (DMEM) and RPMI-1640 Medium were supplied by Gibco Invitrogen. The MCF-7 and bEnd.3 cell lines were ordered from ATCC. Collagen G, Eagle’s medium, L-glutamine, gentamycin, penicillin and streptomycin for cell cultures were purchased from ATCC. Other reagents related to cell experiments were purified and sterilized. All glassware and stirring bars were cleaned with aqua regia (3:1 v/v HCl (37%): HNO₃ (65%) solution) and then rinsed thoroughly with H₂O before use. (Caution: aqua regia solutions are dangerous and should be used with extreme care; never store these solutions in closed containers.) The water in all experiments was prepared in a
three-stage Millipore Milli-Q plus 185 purification system and had a resistivity higher than 18.2 MΩ cm.

2. Synthesis of mesoporous core-shell structured Au@Pt NPs

Typically, 60 mg Brij 58 were dissolved into 8 mL H₂O ultrasonic for about 10 min, followed by the addition of 0.2 mL HAuCl₄ (0.05 M), 0.9 mL K₂PtCl₄ (0.05 M), and 2 mL AA (0.25 M) at room temperature. The color changed from red to black in about 2 min. The mixture was stirred for about 2 hours. Then, the NPs were collected by centrifugation and washed with water three times.

3. Characterization

Transmission electron microscopy (TEM) images were obtained using a Philips CM200 UT (Field Emission Instruments, USA). The tube was operated at 40 KV accelerating voltage and 15 mA current. X-ray diffraction (XRD) characterization was carried out using a Rigaku Miniflex 600. UV-vis spectra were obtained with a Tecan Safire2 microplate reader. Cellular imaging was performed with a CLSM (Leica SP8 Point Scanning Confocal).

4. PEGylation of Au@Pt NPs

For PEG functionalization, 10 mg of mixture of Amino PEG amine and FA PEG amine (ratio of 4:1) was added to 1 mg of Au@Pt NPs dispersed in 2 mL of water. After sonication for 20 min and overnight stirring, excess PEG polymers were removed and the NPs were washed several times with water. The obtained Au@Pt-PEG NPs were highly water-soluble and were stored at 4 °C for future use.
5. Synthesis of TPP-labeled Au@Pt-PEG-FA-Ce6 NPs

TPP-labeled Au@Pt-PEG-Ce6 NPs were obtained by directly coupling carboxyl groups of (4-carboxybutyl) triphenylphosphonium bromide and Chlorin e6 (Ce6) with the amino group on the surface of the NPs to form amino bonds. Au@Pt-PEG NPs with the optimal conjugated ratio were prepared as follows. Briefly, EDC (12 mg mL\textsuperscript{-1}) and NHS (24 mg mL\textsuperscript{-1}) were added to 1 mL of (4-carboxybutyl) triphenylphosphonium bromide (3.5 mg mL\textsuperscript{-1}) and Ce6 (0.5 mg mL\textsuperscript{-1}) mixture solution and stirred for 30 min at room temperature. The reacted mixture was added dropwise into 1 mL of Au@Pt-PEG-FA NPs aqueous solution (1 mg mL\textsuperscript{-1}). After stirring overnight, the crude product was collected and sequentially washed with deionized water and re-dispersed into water for further usage.

UV-vis-NIR spectra before and after drug loading were recorded to determine drug loading ratios. The conjugated amount of Ce6 on the APPF could be determined through the equation:

\[
\psi(\%) = \frac{M_0 - M_y}{M_{AuPt} + M_0 - M_y}
\]

where \( M_0, M_{AuPt} \) and \( M_y \) represent the amounts of total Ce6, NPs and free Ce6, respectively, in the collected supermatant. To measure \( M_y \), Ce6 absorption was recorded at an intensity of 400 nm.

To determine maximal drug loading on NPs, 1 mL of Ce6 solution at various concentrations mixed with 12 mg of EDC and 24 mg of NHS and stirred for 30 min at room temperature. The solution was added to 1 mL of APPF at a concentration of
1 mg mL⁻¹. After 24 h of stirring, the excess unbonded Ce6 was collected and NPs was rinsed with DI water several times. The formed complexes were re-dispersed and stored at 4 °C. The conjugated amount of Ce6 on the NPs could be determined using the equation given above. The absorption peaks at 400 nm were used to determine Ce6 concentrations. Finally, the optimal value was determined to be 10.21%.

6. Cytotoxicity assay

The cytotoxicity of NPs for MCF-7 and bEnd.3 cells in vitro was measured using the standard MTT assay. MCF-7 or bEnd.3 cells at a cell density of 1.0x10⁵ per mL were incubated in 96-well plates for 24 h. Then the medium was discarded and treated with DMEM medium containing NPs at various concentration for 24 h. After that, the cells were incubated with 20 µL of MTT (5 mg mL⁻¹ in PBS) for 4 h. After incubation, 150 µL DMSO was added to each well. The optical density at a wavelength of 490 nm was recorded by a microplate reader.

7. Measurements of oxygen generation

Oxygen concentrations in aqueous solution were measured using a commercial dissolved oxygen meter (ExStik 2, EXTECH, USA). Ten milliliters of deoxygenated water were placed in a flask filled with argon, and 1 mL of Au@Pt NPs (2 mg mL⁻¹) was injected into the flask. The dissolved oxygen meter was inserted into the flask to measure the
oxygen concentration of the solution. Solution without added Au@Pt NPs was measured as a control.

8. Measurements of ROS Generation under Laser Irradiation at 660 nm

The ROS generation of the Au@Pt-PEG-Ce6 NPs was measured using ABDA as an indicator. Briefly, Au@Pt-PEG-Ce6 NPs in PBS (Ce6 amount: 10 µg mL\(^{-1}\)) containing 20 mM ABDA were irradiated with a 660 nm laser with a power intensity of 50 mW cm\(^{-2}\), a 808nm laser with a power intensity of 1.2 W cm\(^{-2}\), or combined 660 nm/808 nm laser irradiation. Afterward, the absorbance change of ABDA was measured using a UV–vis spectrometer.

9. Temperature Elevation Induced by Laser Irradiation at 808 nm

To study the photothermal effect of the Au@Pt-PEG-Ce6 NPs, 2 mL of aqueous solution with various concentrations of the NPs was irradiated with an 808nm laser with a power density of 1.2 W cm\(^{-2}\). The temperature of the solution was monitored using a thermocouple microprobe that was submerged in the solution every 30 s.

10. CLSM Studies of Uptake of Au@Pt-PEG-Ce6 NPs
The uptake of Au@Pt-PEG-Ce6 NPs by MCF-7 cells was investigated using CLSM. MCF-7 cells (1 × 10^5) were used to seed 35-mm glass-bottom Petri dishes and cultured for 24 h at 37 °C in an incubator. The Au@Pt-PEG-Ce6 NPs were then added to the cells and further incubated for 2 h. Subsequently, the MCF-7 cells were washed three times with PBS (pH 7.4) and then fixed with 4% paraformaldehyde (PFA) for 15 min. Finally, the cells were imaged using CLSM with a laser excitation at 543 nm for Ce6.

11. Mitochondrial uptake of nanosensitizers

MCF-7 cells were incubated in 96-well plates at a cell density of 1.0x10^5 per mL for 24 h. Then the medium was discarded and treated with DMEM medium containing NPs (Ce6 amount: 10 µg mL^{-1}) for 12 h. After that, the cells were washed with PBS and stained with MitoTracker Green (30 nM) for 15 min. Then the cells were washed with PBS three times. The cells were observed using CLSM and imaging was done at a wavelength of 488 nm.

12. Cellular internalization pathways

MCF-7 cells were incubated in 96-well plates at a cell density of 1.0x10^5 per mL for 24 h. The cancer cells were incubated with various kinds of inhibitors including ethylisopropylamiloride (EIPA) (100 µM), chlorpromazine (20 µM), dynasore (50 µM), and filipin (10 µM) for 30 min. Then the medium was discarded and treated with DMEM
medium containing NPs at a concentration of 100 µg mL⁻¹ for 12 h. After that, the cells were washed with PBS and CLSM images were taken.

13. ROS burst in vitro

Six groups of MCF-7 cells were used to seed a CLSM dish and incubated for 24 h. Au@Pt-PEG-Ce6 NPs were added to the cells in DMEM culture medium and kept there for 12 h. Then, the cells were washed with PBS buffer three times and fresh DMEM medium containing 10% fetal bovine serum was added. The six groups of cells were irradiated with a 660 nm laser for 0, 30, 60, 90, 120, and 150 s, respectively. Subsequently, the cells were incubated with SOSG kits and cultured for 12 h. Then the cells were examined with CLSM at a wavelength of 488 nm. A real-time ROS monitoring experiment was also carried out. Eight groups of cells were irradiated with a 660 nm laser for 150 s. Subsequently, the cells were examined with CLSM with a 488 nm excitation at 2 h intervals.

To investigate the peroxidase-like activity of Au@Pt NPs, catalysis to peroxidase substrate TMB was tested in the presence of H₂O₂. The relative reaction is described in Figure S2: in equation 1, H₂O₂ is catalyzed to oxygen by Au@Pt NPs, and in equation 2, oxygen serves as an electron donor for the oxidation of TMB under an acidic condition, resulting in a deep blue color of the solution. The concentration-dependent color intensity was also verified (Figure S4 a-b). An easily recognized blue color was observed even for a low concentration of Au@Pt NPs. This indicates that Au@Pt NPs behave as peroxidase
toward TMB oxidation with \( \text{H}_2\text{O}_2 \). These reactions are ascribed to the charge-transfer complexes derived from the one-electron oxidation of TMB.

On the basis of the peroxidase-like activity of Au@Pt NPs, the relationship between the peroxidase-like activity of Au@Pt NPs and \( \text{H}_2\text{O}_2 \) concentration was further studied (Figure S3). It was clear that the absorbance strongly depended on the \( \text{H}_2\text{O}_2 \) concentration: it increased linearly (red line) in the concentration range from \( 5 \times 10^{-6} \) to 10 mM. Hence, our methodology provides a visual detection method without any complicated instrumentation. The designed Au@Pt NPs exhibit extremely strong peroxidase-like activity at low concentrations and therefore hold great potential for effective oxygen production.
Figure S1. TEM image of as-obtained mesoporous core-shell structured Au@Pt NPs.
Figure S2. Digital photographs for the dispersion status of APPF NPs (left) and after store for one month (right). Concentration = 1 mg mL$^{-1}$. 
Figure S3. Zeta potential values for Au@Pt, APP and APPTFC.
Figure S4. (a) The equation of peroxidase-like reaction between Au@Pt NPs and H$_2$O$_2$.
(b) The equation of peroxidase-like reaction between Au@Pt NPs and TMB in acidic solution.
Figure S5. (a) Photographs of different concentrations of Au@Pt NPs consisting of 10 mM H$_2$O$_2$ and 1 mM TMB: (1) 2 μg mL$^{-1}$, (2) 10 μg mL$^{-1}$, (3) 25 μg mL$^{-1}$, (4) 50 μg mL$^{-1}$. (b) Comparison of the absorbance evolution at 652 nm for different concentration of Au@Pt NPs. The higher the absorbance, the more the oxidation of TMB.
Figure S6. The calibration curve plotted on peroxidase-like reaction with different concentration of $\text{H}_2\text{O}_2$. All the conditions: 20 mM TMB, 5 mM $\text{H}_2\text{O}_2$, 0.2 mM HAc-NaAc buffer (pH 4.5) and 10 $\mu$g mL$^{-1}$ Au@Pt NPs.
Figure S7. (a) Photos and (b) quantitative dissolved oxygen generation of APPF NPS after reaction with increasing concentrations of $\text{H}_2\text{O}_2$. 
Figure S8. Absorbance of 9,10-dimethylanthracene (ABDA, 20 mM) after photodecomposition by ROS generation upon NIR laser irradiation in the presence of the (a) APPTFC nanosystem in PBS (660 nm). (b) APPTFC nanosystem in PBS (808 nm); (c) APPTFC nanosystem with H$_2$O$_2$ at acid solution (660 nm).
Figure S9. (a) The normalized absorbance of 9,10-dimethylanthracene (ABDA, 20 mM) at 380 nm during photodecomposition by ROS generation in the presence of Au@Pt-PEG NPs in PBS (660 nm), APPTFC nanosystem in PBS (808 nm), APPTFC nanosystem in PBS (660 nm), APPTFC nanosystem in PBS (660 nm + 808 nm), free Ce6 in PBS (660 nm) and APPTFC nanosystem in water (660 nm), respectively. (b) Normalized absorbance of 9, 10-imethylanthracene (ABDA, 20 mM) at 380 nm during photodecomposition by ROS generation in presence of Au@Pt-PEG-Ce6 NPs and free Ce6.
Figure S10. Confocal images of MCF-7 cells and A549 cells incubated with APPTFC for 12 h.
Figure S11. Quantification of APPTFC incubated in MCF-7 cells and A549 cells after 12 h. Data was expressed as mean ± SD from triplicate samples.
Figure S12. The relative fluorescence intensity of MCF-7 cells without (control) or with various inhibitors in Figure 3c.
Figure S13. The quantification of fluorescent intensity of the line scanning profiles in the corresponding confocal images.
Figure S14. CLSM images of CellLight late Endosomes-GFP stained endosome, APPTFC and the overlay channel.
Figure S15. CLSM images of MTG, Au@Pt@PEG-FA-Ce6 without TPP (APPFC) and the overlay channel.
Figure S16. *In vitro* evaluation of oxygen generation by Au@Pt NPs.
Figure S17. Relative fluorescence intensity of MCF-7 cell incubated with APPTFC nanosystem upon irradiation for different time.
Figure S18. MCF-7 cells were incubated with the APPTFC nanosystem and irradiated for different period of time. Then the cells were incubated with rhodamine 123. CLSM images of rhodamine 123-stained cells were obtained by excitation of the samples at 488 nm.
Figure S19. Relative fluorescence intensity of rhodamine 123-stained cells with different irradiation time.
Figure S20. Cell viability of MCF-7 cells incubated with the APPTFC nanosystem without TPP modification upon irradiation for different period of time.