Supporting Information

Gold Cube-in-Cube Based Oxygen Nanogenerator: A Theranostic Nanoplatform for Modulating Tumor Microenvironment for Precise Chemo-Phototherapy and Multimodal Imaging

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<th>Abbreviation</th>
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<tr>
<td>CC</td>
<td>gold cube-in-cube</td>
</tr>
<tr>
<td>CCm</td>
<td>gold cube-in-cube@mSiO$_2$</td>
</tr>
<tr>
<td>CCmMC</td>
<td>gold cube-in-cube@mSiO$_2$@Mn-Cdot</td>
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<tr>
<td>RGD-CCmMC/DOX</td>
<td>RGD-conjugated CCmMC loaded with DOX</td>
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Reagents and Materials

All chemicals were obtained from commercial suppliers and used without further purification. The tetrachloroauric(III) acid (HAuCl₄·3H₂O), ascorbic acid (AA), silver nitrate (AgNO₃), Cetyltrimethylammonium bromide (CTAB), ammonia aqueous solution, Sodium borohydride, cetyltrimethylammonium chloride (CTAC), were obtained from Sigma-Aldrich. Triethylamine (TEA), tetraethyl orthosilicate (TEOS), 3-Amino-propyl-triethoxysilane (APTES), Doxorubicin hydrochloride (DOX) were obtained from Aladdin Chemistry, Co.,Ltd. Absolute ethanol, sodium hydrate (NaOH), ammonium nitrate (NH₄NO₃), hydroxide (H₂O₂) were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Cell counting kit assay kit (CCK-8), Singlet Oxygen Sensor Green reagent (SOSG), 2’,7’-Dichlorodihydrofluorescein diacetate (DCFH-DA) and Live-Dead Cell Staining Kit were obtained from Beyotime Institute of Biotechnology (Shanghai, China).

Instruments

The morphology and structure of as-prepared nanoparticles were characterized using transmission electron microscopy imaging (FEI Tecnai G2 Spirit Twin, Holland). The scanning transmission electron microscopy (STEM) and elemental mapping images were obtained to the elemental composition. The crystal structure of the sample was determined by X-ray diffractometer (Bruker D8 Advance) with Cu Kα radiation. UV-vis absorption spectra of different samplesn were recorded by an Evolution 220 UV-Visible Spectrophotometer (Thermo Fisher Scientific). Nitrogen adsorption-desorption measurements were performed on a micromeritics instrument (ASAP2020). The pore size distribution was calculated by the Barrett-Joyner-Halenda (BJH) method. X-ray photoelectron spectroscopy (XPS) was evaluated using X-ray photoelectron spectrometer (Thermo Scientific Escalab 250Xi). Flow cytometry was carried out by using BD LSRFortessa flow cytometer. The hydrodynamic diameters and zeta potential were determined using a Malvern ZetaSizer Nano-ZS instrument. High-resolution TEM (HRTEM) images were recorded with a FEI-TECNAI G2 transmission electron microscope operating at 200 kV. The surface morphology was characterized using atomic force microscopy (AFM, Veeco’s MultimodeV, Veeco, USA).
Hemolysis Assay

Red blood cells were separated and collected through centrifugation and purified several times with PBS buffer to remove sera from the human blood after stabilization by EDTA.K2. Then, the purified blood cells were diluted to 10 times with PBS solution (pH = 7.4). 0.3 mL of diluted cells suspension was then mixed with (i) 1.2 mL of deionized water as a positive control; (ii) 1.2 mL of PBS as a negative control; and (iii) 1.2 mL of materials suspensions with varying concentrations (6.25, 12.5, 25, 50, 100, 200, 400, 800 and 1000 μg/mL). Subsequently, all the mixtures were gently shaken and kept for 3 h at 37 °C. Finally, the mixtures were centrifuged and the absorbance of the upper supernatants was measured by UV-vis. The percentage of hemolysis was calculated using the following equation.

\[
\text{Hemolysis \%} = \frac{[A_{\text{sample}} - A_{\text{control(-)}}]}{[A_{\text{control(+)}} - A_{\text{control(-)}}]}
\]

Animals Tumor Model

Four to six-week old female Balb/c nude mice were purchased from the Animal Model Research Institute of Nanjing University, and all procedures and experimental protocols were in accordance with the guidelines of the Institutional Animal Care and Use Committee. Tumor models were established by subcutaneous injection of 4T1 cells (2×10^6 in PBS, 100 µL) into the right shoulder of female nude mice. During the process, we guarantee that the number of cells, the position of subcutaneous injection, and the volume of the cell solution are exactly identical. Tumor diameters were measured every other day with a Vernier caliper in 2D and tumor volumes were calculated by the following formula. When the tumor grew to 8-10 mm in diameter, the mice were used for the following in vivo experiments.

\[
\text{tumor volume} = \text{length} \times \text{(width)}^2 / 2
\]

Detection of Intracellular O2 Generation in 4T1 Cell
The intracellular O$_2$ levels was determined by RDPP probe, [(Ru(dpp)$_3$)]Cl$_2$, an O$_2$ sensing probe, whose fluorescence can be strongly quenched in presence of O$_2$ molecule. 4T1 cells were incubated and treated with CCmMC (50 µg/mL) overnight at 37 °C. Afterwards, the medium was replaced and 4T1 cells were incubated with new medium containing 10 μM RDPP for 4 h. Then the intracellular fluorescence of RDPP was excited at 488 nm, and observed by confocal laser scanning microscopy. Besides, to further demonstrate intracellular generation of O$_2$, 4T1 cells were also incubated with CCmMC suspension (50 µg/mL in DMEM) at 37 °C for various times (20 min, 1 h, and 3 h) and further cultured with 10 μM RDPP medium.
Figure S1. (a) Hydrodynamic diameters of CCmMC measured via dynamic light scattering (DLS); (b) Zeta potential values of different nanoparticle solutions in water.
Figure S2. Characterization of as-synthesized Mn-Cdots. (a) TEM image of monolayer Mn-Cdot; (b) HRTEM image of Mn-Cdot; (c) The AFM image of monolayer Mn-Cdot. Inset in (c): Height profile along the corresponding line; (d) the hydrodynamic diameter of Mn-Cdot; (e) XPS spectra of Mn-Cdot. The inset showed high-resolution XPS spectra of Mn 2p for Mn-dot.
Figure S3. XPS spectra shows the Mn, O, C and Au characteristic peaks for CCmMC nanocomposites. The inset showed high-resolution XPS spectra of Mn 2p for the CCmMC.
**Figure S4.** The UV-vis absorption spectra of gold nanocubes and gold cube-in-cubes.
Figure S5. The pore size distribution profile of CCm and RGD-CCmMC/DOX NPs.
**Figure S6.** Plot of cooling time as a function of the negative natural logarithm of the temperature driving force obtained from a cooling stage. The time constant, $\tau_s$, is determined to be 285.4 s.

The photothermal conversion efficiency ($\eta$) of CCmMC can be calculated as following equations:

$$\eta = \frac{hS (T_{\text{max}} - T_{\text{surr}}) - Q_{\text{dis}}}{I (1 - 10^{-A_{808}})} \times 100\%$$  \hspace{1cm} (1)$$

where $h$ is the heat transfer coefficient; $S$ is the irradiated area; $T_{\text{max}}$ is the equilibrium temperature; $T_{\text{surr}}$ is ambient temperature of the surroundings; $I$ is the laser power density (1 W cm$^{-2}$); $Q_{\text{dis}}$ is the baseline energy input from the light absorption by the solvent; $A_{808}$ nm is the absorbance of the CCmMC NPs at 808 nm. Where $h$ (W cm$^{-2}$ K$^{-1}$) means heat transfer coefficient, $S$ (cm$^2$) represents the surface area of the container. A dimensionless driving force temperature ($\theta$) is introduced to calculate the value of $hS$ using the following equations:

$$\theta = \frac{T - T_{\text{surr}}}{T_{\text{max}} - T_{\text{surr}}}$$  \hspace{1cm} (2)$$

$$t = -\tau s \ln \theta$$  \hspace{1cm} (3)
\[ \tau_s = \frac{\sum m_i C_{p,i}}{hS} \]  

Where \( m_i \) and \( C_{p,i} \) is the mass (1.0 g) and the thermal capacity (4.2 J·g\(^{-1}\)·\(^{\circ}\)C\(^{-1}\)) of deionized water used as a solvent, \( t \) is cooling time after irradiation, and \( \tau_s \) is the sample system time constant. On the basis of the above equation, the photothermal conversion of the CCmMC was determined to be about 65.6% under irradiation by an 808 nm laser, which is much higher than that of gold nanocube (37.2%).
**Figure S7.** The temperature change in Au cube-in-cube and Au cube aqueous solution (400 µg mL\(^{-1}\)) in response to NIR laser on and off at a duration of 1200 s.
Figure S8. Cumulative DOX release from RGD-CCmMC/DOX nanovehicles under different pH values (5.0, 6.6, 7.4) at 37 °C and 50 °C.
Figure S9. Detection of intracellular O$_2$ levels. (a) Intracellular O$_2$ level after treated with different formulations via CLSM observation. Scale bars: 20 µm. (b) Corresponding fluorescence intensity in cells with different formulations. (c) Intracellular O$_2$ detection at various time period (20 min, 1 h, and 3 h). Scale bars: 20 µm.
**Figure S10.** Flow cytometry detection of ROS generation using DCFH-DA of 4T1 cells treated with Saline, Mn-Cdots, CCmMC and CCmMC+H\textsubscript{2}O\textsubscript{2} with 635 nm laser irradiation.
Figure S11. (a) Cell viability after treated with various concentrations of CCmMC or RGD-CCmMC NPs determined by CCK-8 assay of 4T1 cells. (b) Cell viability after treated with various concentrations of Au nanocube or Au cube-in-cube NPs. Each value represents the mean ± SD of three replicates.
Figure S12. (a) ICP quantitative analysis of the Au content in feces at 2 h, 8 h, 24 h, 3 d, 5 d and 7 d after injection. (b) ICP quantitative analysis of the Au content in blood at different time points. Data are presented as mean ± SD (n = 3).
Reference


