Hypoxia-responsive and uperconversion nanoparticles modified RBC micro-vehicles fabrication for oxygen delivery and chemotherapy enhancement

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Part A: Experimental Section

1. Materials and characterization

Materials. All solvents used were of analytical grade without further purification. Gadolinium (III) chloride anhydrous ($GdCl_3$, 99.99 %), ytterbium (III) chloride anhydrous ($YbCl_3$, 99.9 %), thulium (III) chloride anhydrous ($TmCl_3$, 99.9 %), sodium trifluoroacetate (Na-TFA, 98 %), 1-octadecene (ODE, 90 %), oleic acid (OA, 90 %), Rose Bengal and hexanoic acid were supplied by Sigma Aldrich. Poly (ethylene glycol)–distearoyl phosphatidylethanolamine, (DSPE-PEG_{2000}-NH_2) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG_{2000}-biotin) were purchased from Avanti Polar Lipids. The FSH (Arg-Gly-Asp) peptides were purchased from Shanghai Qiangyao Biotechnology Co., Ltd.

Characterization. Transmission electron microscopy (TEM) measurements were carried out on a JEM 2100F microscope (Japan) operated at 200 kV. The samples were first dispersed in ethanol and then collected by using copper grids covered with carbon films for measurements. Scanning electron microscopic (SEM) images were obtained on a Philip XL30 microscope (Germany). A thin gold film was sprayed on the samples before characterization. UV–vis–NIR absorption spectra were measured on a Shimadz spectrophotometer (UV-3150) (Japan) with wavelength range of 300-1200 nm. The fluorescence spectra were recorded on Edinburgh Fluorescence Spectrometer FLS980 instrument with excitation source were using external 980-nm semiconductor laser

(Changchun New Industries Optoelectronics Tech. Co., Ltd.), unless otherwise specified, all spectra were collected under identical experimental conditions. Visible Fluorescence spectra were recorded on Edinburgh Fluorescence Spectrometer FLS980 instrument with Xenon lamp as excitation source.

2. Synthesis of lanthanide doped core-shell structured $NaGdF_4$:Yb, Tm@$NaGdF_4$ up-conversion nanoprobe.

Preparation of shell precursors for the synthesis of up-conversion nanoparticles

Gd-OA (0.10 M) host precursor: a mixture of $GdCl_3$ (2.50 mmol), OA (10.0 mL), and ODE (15.0 mL) was loaded in a reaction container and heated at 140 °C under vacuum with magnetic stirring for 30 min to remove residual water and oxygen. Then the colorless Gd-OA precursor solution (0.10 M) was obtained.

Yb-OA, Tm-OA (0.1 M): The synthesis of Yb-OA and Tm-OA precursor was carried out all the same as that of Gd-OA except 2.50 mmol of $YbCl_3$, 2.50 mmol of $TmCl_3$, were used instead of 2.5 mmol of $GdCl_3$, respectively.

Na-TFA-OA (0.20 M) precursor: A mixture of Na-TFA (2.00 mmol) and OA (10.0 mL) was added into a container at room temperature under vacuum with magnetic stirring to remove residual water and oxygen. Then the colorless Na-TFA-OA precursor solution (0.20 M) was obtained.

Synthesis of $NaGdF_4$:Yb,Tm@$NaGdF_4$ UCNPs with a diameter of 40 nm: in a typical procedure for the synthesis of NaGdF4: 20 % Yb, 0.3 % Tm nanocrystals, 0.797 mmol of anhydrous $GdCl_3$, 0.20 mmol of $YbCl_3$ and 0.003 mmol of $ErCl_3$ were added

to a 100-mL flask containing 10 mL of oleic acid and 15 mL of 1-octadecene. The mixture was heated at 150 °C for 30 min before cooling down to 50 °C to remove the water content from the solution. Shortly thereafter, 10 mL of methanol solution containing NH_4F (2.75 mmol) and NaOH (2.5 mmol) was added and the resultant solution was stirred for 30 min to remove the methanol. After the methanol was evaporated, the solution was heated to 300 °C under argon for 1 h and then cooled down to room temperature. The resulting 15-nm $NaGdF_4$:Yb,Tm nanoparticles were precipitated by addition of ethanol, collected by centrifugation at 6000 rpm for 5 min, washed with ethanol several times, and re-dispersed in 10 mL of cyclohexane. Then, 2.5 mL of the purified $NaGdF_4$:Yb,Tm initial core solution was mixed with 4.0 mL of OA and 6.0 mL of ODE. The flask was pumped at 70 °C for 30 min to remove cyclohexane and residual air. Subsequently, the system was switched to air flow and the reaction mixture was further heated to 280 °C at a rate of ~ 20 °C/min. Then Gd-OA (0.1 M, 1.0 mL) and Na-TFA-OA (0.20 M, 1 mL) host shell precursors were alternately introduced by dropwise addition at 280 °C and the time interval between each injection was 15 min. There are eight groups of Gd-OA and Na-TFA-OA. Finally, the obtained $NaGdF_4$:Yb,Tm@$NaGdF_4$ homogeneously doping cores were centrifuged and washed as above and dispersed in cyclohexane.

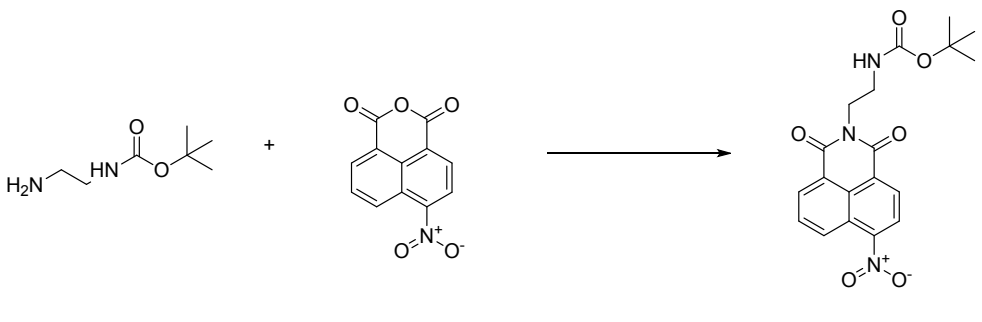
3. Synthetic procedures of hypoxia probe

tert-butyl(2-aminoethyl)carbamate (5)

5

1,2-Diaminoethane (11.95 g, 198.77 mmol) was added into a 1000 mL round-bottomed flask containing dichloromethane (50 mL). Di-tert-butyldicarbonate (7.23 g, 33.13 mmol), pre-dissolved in dichloromethane (400 mL), was added slowly to the above reaction mixture under stirring at room temperature over a period of 6 h. After reacting for 12 h, the solution was filtered to remove the resulting white precipitate. Excess 1,2-diaminoethane and 1,4-dioxane was removed under vacuum. The residue was then dissolved in (60 mL) ethyl acetate, washed by saturated Na_2CO_3 (3 * 40 mL). Anhydrous sodium sulfate was added to dry the organic layer followed by filtration. Then the solvent was removed by rotary evaporation and the residue was dried under vacuum, to give colorless oil which was then purified by column chromatography to afford compound **5** (5.2 g, 97.7 % yield). ^{1}H NMR ($CDCl_3$, 400 MHz), δ (ppm): 4.89 (s, 1H), 3.19 (m, 2H), 2.82 (m, 2H), 1.53 (s, 2H), 1.46 (s, 9H).

tert-butyl(2-(6-nitro-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)ethyl)carbamat-e (4)



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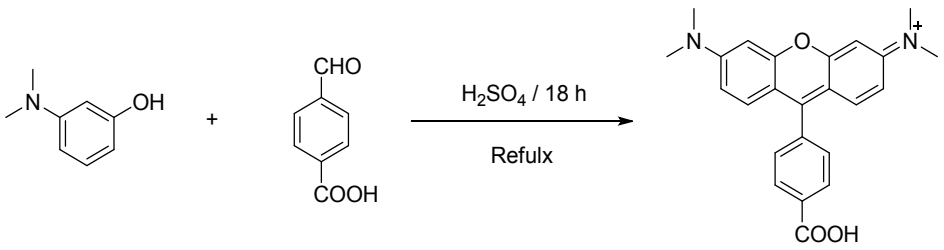
Compound **5** (160 mg, 1.0 mmol) was added to the solution of 4-nitro-1,8-naphthalic anhydride (125 mg, 0.5 mmol) in 10 mL ethanol. The mixture was refluxed for 3 h and the suspended 4-nitro-1,8-naphthalic anhydride dissolved slowly during the process of the reaction. The reaction mixture was cooled to room temperature and removed the solvent by filtration to afford the desired compound **3** (185 mg, 96.0 %) as yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.83 (d, 1H, J = 8.0 Hz,), 8.74 (d, 1H, J = 8.0 Hz), 8.70 (d, 1H, J = 8.0 Hz), 8.41 (d, 1H, J = 8.0 Hz), 7.99 (t, 1H, J = 8.0 Hz), 4.93 (br, 1H), 4.38 (t, 2H, J = 5.6 Hz), 3.50–3.60 (m, 2H), 1.25 (s, 9H).

N-(9-(4-carboxyphenyl)-6-(dimethylamino)-3H-xanthen-3-ylidene)-N-methylmethanaminium (2)

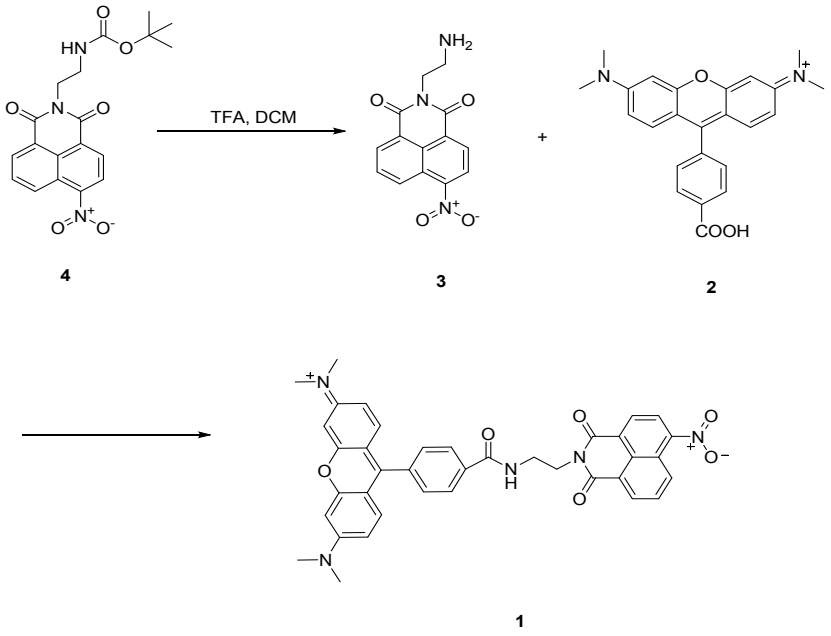


2

4-formylbenzoic acid (5.472 g, 36.44 mmol) was added to a stirred solution of concentrated sulfuric acid (55 mL) and deionized water (40 mL) at room temperature. The mixture was then heated to 180 C within 2 hours under nitrogen atmosphere, during which 3-(dimethylamino) phenol (10.063 g, 73.45 mmol) was slowly added. After refluxed for 18 h at 180 C, the solution was cooled to r.t. and poured into ice water. NaOH solution (5 M) was added to adjust the pH of the mixture about 6-7 to obtain a

large amount of precipitate. The crude product was filtered, washed with water to get the purple crude product which was then dissolved in 200 mL ethanol. The solution was filtered to remove the insoluble inorganic salt. After removal of ethanol under vacuum, the residue was purified by column chromatograph to give compound **2** as orange powder which was used in the next reaction without further purification.

N-(6-(dimethylamino)-9-(4-((2-(6-nitro-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)ethyl)carbamoyl)phenyl)-3H-xanthen-3-ylidene)-N-methylmethanaminium (1)



Compound **4** (185 mg, 0.48 mmol) was dissolved in dichloromethane (10 mL), to which trifluoroacetic acid (TFA, 20 mL) was slowly added at 0 ºC. The solution was then reacted for 1.5 h at room temperature. After reaction, excess TFA was removed under vacuum and NaOH solution was added to adjust the pH of the mixture to 7-8. The residue was then extracted by dichloromethane, dried with anhydrous sodium sulfate and concentrated to compound **3,** which was directly used in the next reaction.

Compound **2** (186 mg, 0.48 mmol) was dissolved in 10 mL DMF containing EDCI (468 mg) and HOBT (308 mg). Compound **3** (137 mg, 0.48 mmol) pre-dissolved in DMF (6 mL) was added into the above reaction mixture, which was then stirred at 50 ºC for 12 hours. After that, DMF was removed under vacuum and the residue was purified by column chromatograph to give the final compound **1** as purple solid (245 mg, 78 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.79 (d, 1H, J = 8.0 Hz,), 8.73 (d, 1H, J = 4.0 Hz), 8.68 (d, 1H, J = 8.0 Hz), 8.53 (m, 1H), 8.36 (d, 1H, J = 8.0 Hz), 8.08 (d, 2H, J = 8.0 Hz), 7.93 (t, 1H, J = 8.0 Hz), 7.38 (d, 4H, J = 8.0 Hz), 6.93 (d, 2H, J = 8.0 Hz), 6.81 (d, 2H, J = 4.0 Hz), 4.58 (m, 2H), 3.98 (m, 2H), 3.32 (s, 12H). TOF-MS: $[M]^+$ 654.2347; found 654.2349.

4. Oxygen release *in vitro*

Cell viability: $CaOV_3$ cells were cultured in DMEM low glucose medium containing 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin at 37 °C under 5 % CO_2. Hypoxic Condition for $CaOV_3$ cell line was prepared under 93 % N_2 and 2 % O_2 in incubator. To carry out photodynamic therapy of cancer cells, $CaOV_3$ cells were collected through centrifugation and diluted to a density of 1* 10^4 cells/mL in the DMEM low glucose medium and then seeded onto 96-well plates (100 μL per well). After 24 h culturing, RBCmv was added to the culture medium with five parallel wells for each concentration (0 ~ 500 μg/mL). Finally, after 24 h incubation, biocompatibility was evaluated by CCK-8 assay. For elevating O_2 release for chemotherapy enhancement experiment, a power adjustable 980-nm fiber laser was

collimated and employed as area light source to irradiate the $CaOV_3$ cells after cellular uptake of RBCmv with 4 h in hypoxia condition. After 10 min exposure of 980-nm light at different power densities, the cells were allowed to incubate for an additional 2 h. Cell viability was measured by a CCK-8 kit. Cell viability of PTX, PBS and only RBCmv was also evaluated.

Dissolved O_2 detection: The △dissolved O_2 was detected by dissolved oxygen meter. RBCmv (100 μg/ml) and RBC were dispersed in hypoxia simulate buffer, respectively. Then they were irradiated with 980-nm laser for different times. RBCmv in normoxia simulated buffer and RBCmv- in hypoxia condition were also carried out at the same time. Finally, the solution was collected and sealed for the dissolved O_2 concentration detection with dissolved oxygen meter.

PI staining of $CaOV_3$ cells apoptosis/necrosis: For apoptosis/necrosis observation, $5*10^4$ cells were seeded in cell culture dish, after 24 h culturing in hypoxia condition, RBCmv (250 μg/ml) was added into fresh medium and incubated for another 12 h. Then cells were treated with 980-nm NIR laser for 10 min. Finally, after another 2 h incubation, cells were stained by PI for 30 min and observed by CLSM. Other CLSM images of tumor cells after different treatments were also studied by PI staining and CLSM observing under the same conditions.

5. Photoacoustic imaging *in vivo*

Animal model: All the animal procedures were in agreement with the guidelines of the Institutional Animal Care and Use Committee of Chinese Academy of Science

and performed in accordance with institutional guidelines on animal handling. $CaOV_3$ mouse tumor was sheared into tissue blocks with size as 1mm*1mm*1mm and resuspended in sterile PBS. These tissues were implanted subcutaneously into the right arm of 5 weeks old female nude mice.

***Photoacoustic imaging in vivo*:** The vascular saturated oxygen was evaluated by multispectral photoacoustic imaging on the VisualSonics LAZR and Vevo 2100 Photoacoustic CT Scanner. After exposed with 980-nm NIR for 10 min, oxygenated hemoglobin and total hemoglobin were measured at excitation wavelength of 850 and 750 nm after injected with pure RBC (200 μL) and RBCmv (8 mg/mL, 200 μL). The vascular saturated oxygen of another two groups of RBCmv and pure RBC without NIR laser exposure were also carried out under the same conditions.

Part B: Supplementary Figures

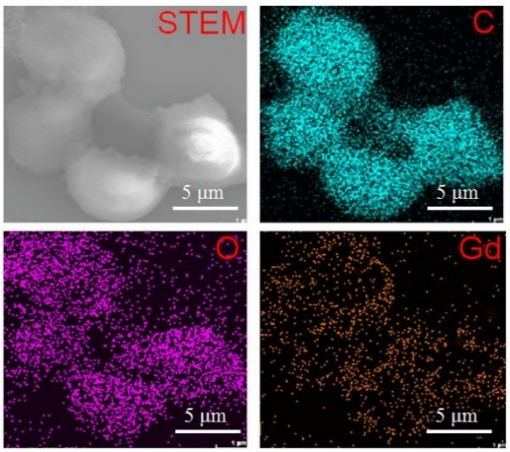


Figure S1 Elemental mapping results of RBC micro-vehicles. The samples were dehydrated by ethanol with different concentration.

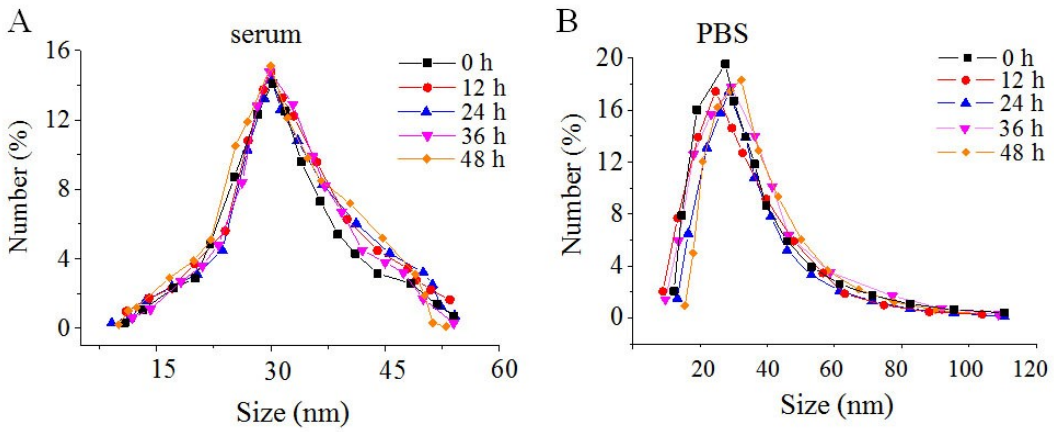


Fig. S2 Size distribution of RBCmv incubated in PBS (A) and Plasma (B) for different hours.

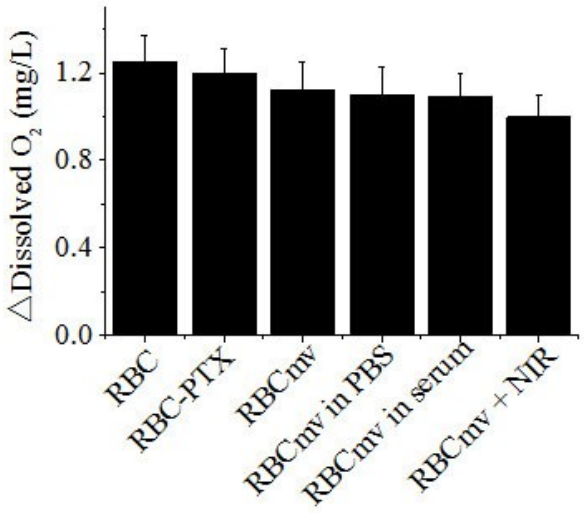


Fig. S3 The △dissolved O_2 concentration of RBCs in different samples after stepwise functionalization.

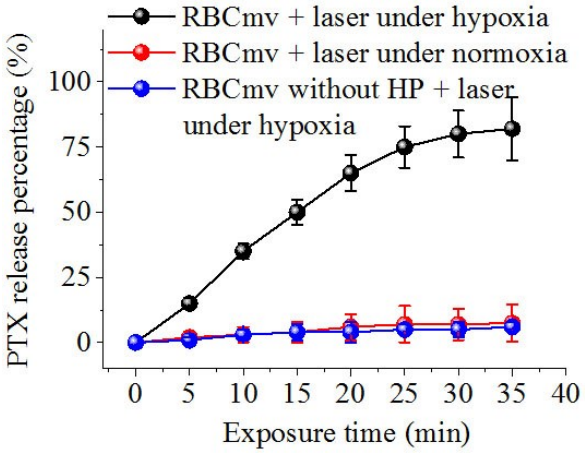


Figure S4 *In vitro* estimation of PTX release of RBCmv in hypoxia and normoxia stimulated buffer fluid, RBCmv without HP fabrication in simulated hypoxia as the function of 980-nm laser irradiation time.

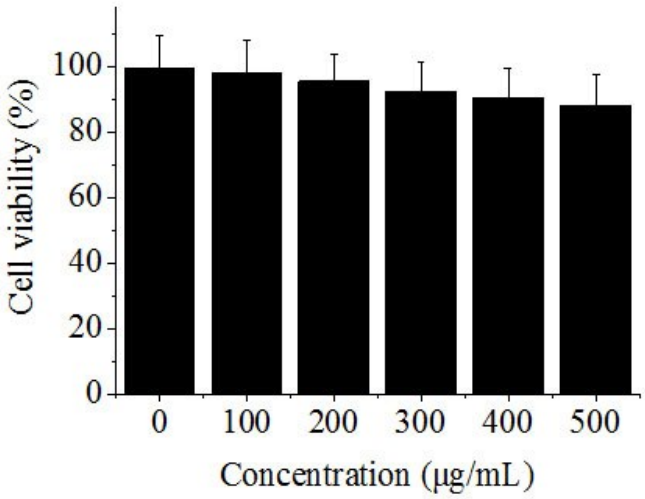


Fig. S5 Cell viability of $CaOV_3$ after incubating with RBCmv with different concentration for 24 hours.

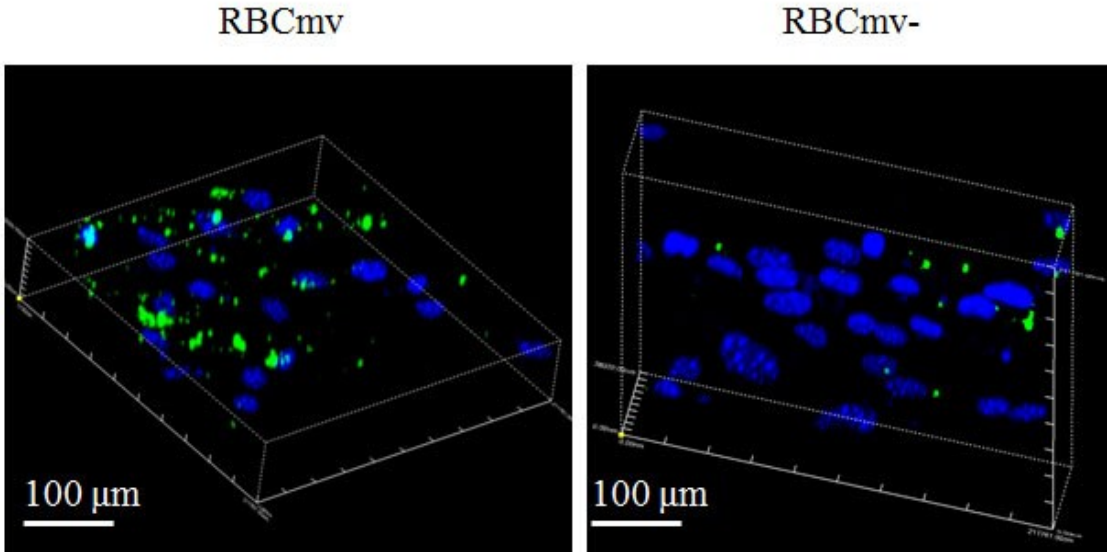


Fig. S6 3D CLSM images of hypoxia $CaOV_3$ cells after co-incubation with RBCmv and RBCmv-.

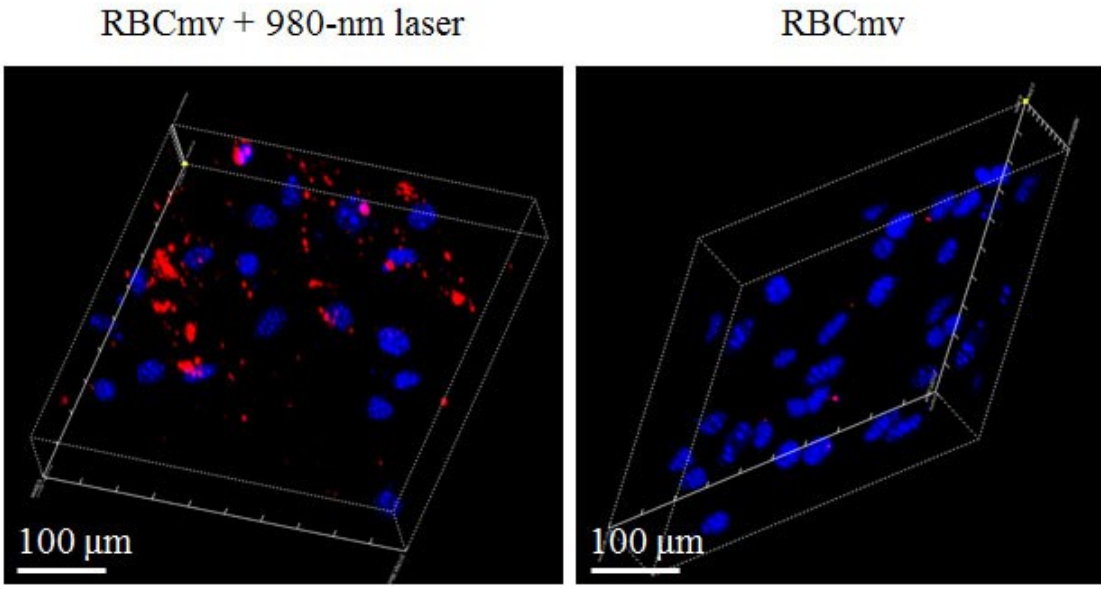


Fig. S7 3D CLSM images of O_2 release in hypoxia $CaOV_3$ after co-incubation of RBCmv with and without exposure to 980-nm laser for 10 min.

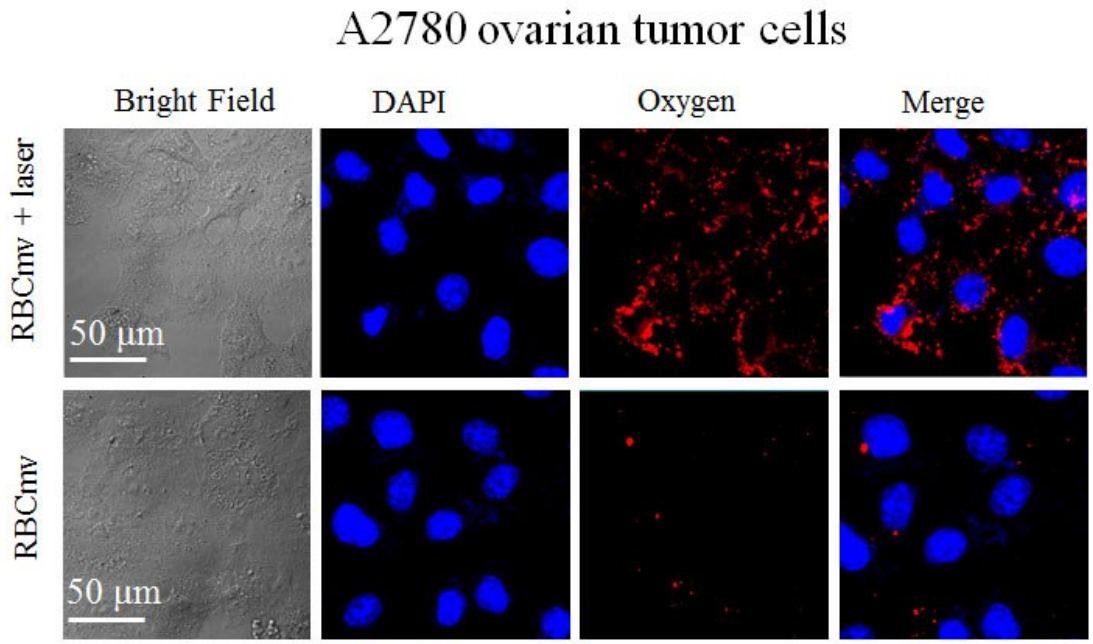


Fig. S8 O_2 release in hypoxic A2780 ovarian tumor cells after incubation with RBCmv for 4 h. Tris(4,7-diphenyl-1,10-phenanthroline) ruthenium(II) dichloride ($Ru(dpp)_3^{2+}$) was the oxygen indicator which can be quenched by O_2. CLSM images in RBCmv + laser group were obtained after exposure to 980-nm NIR laser for 10 min.

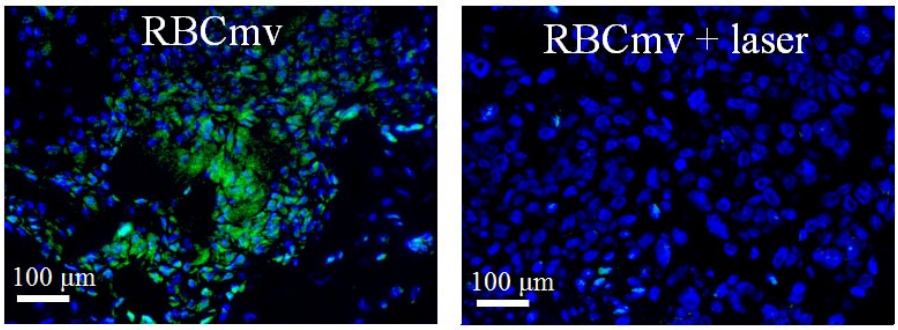


Figure S9 HIF-1α expression detected by immunofluorescence staining after RBCmv incubation with/without 980-nm laser irradiation for 30 min.

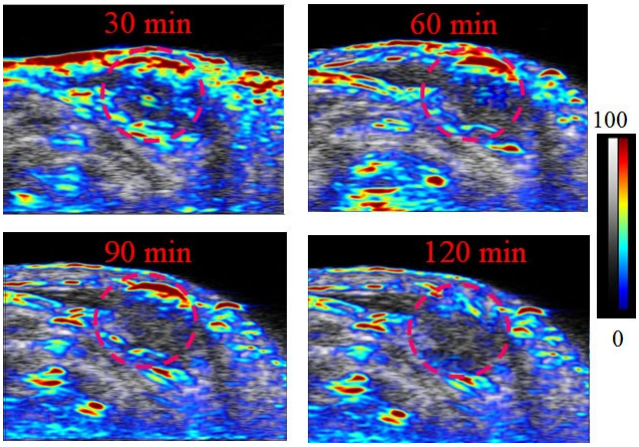


Figure S10 Photoacoustic images of vascular saturated O_2 in $CaOV_3$ subcutaneous solid tumors after different minutes of 980 nm laser irradiation at 12 h post-injection of RBCmv.

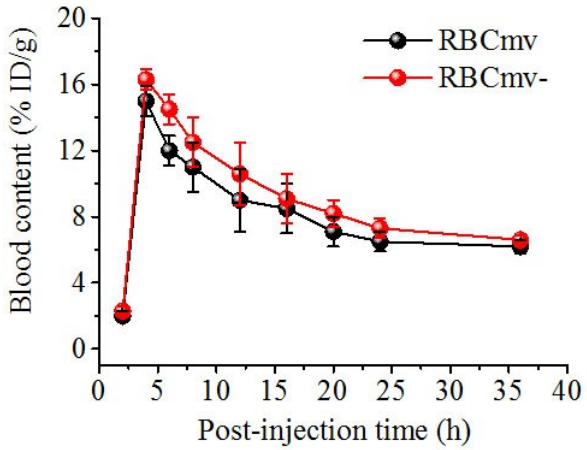


Figure S11 Blood distribution investigation after intravenous injection of RBCmv and RBCmv- for various periods.

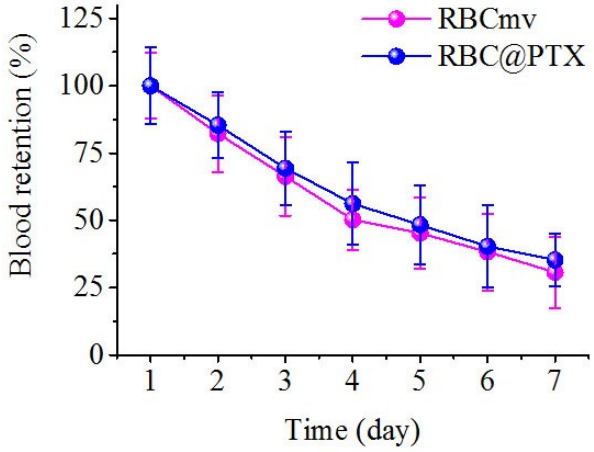


Figure S12 Blood retention of RBCmv and RBC@PTX at different days after the tail injection.

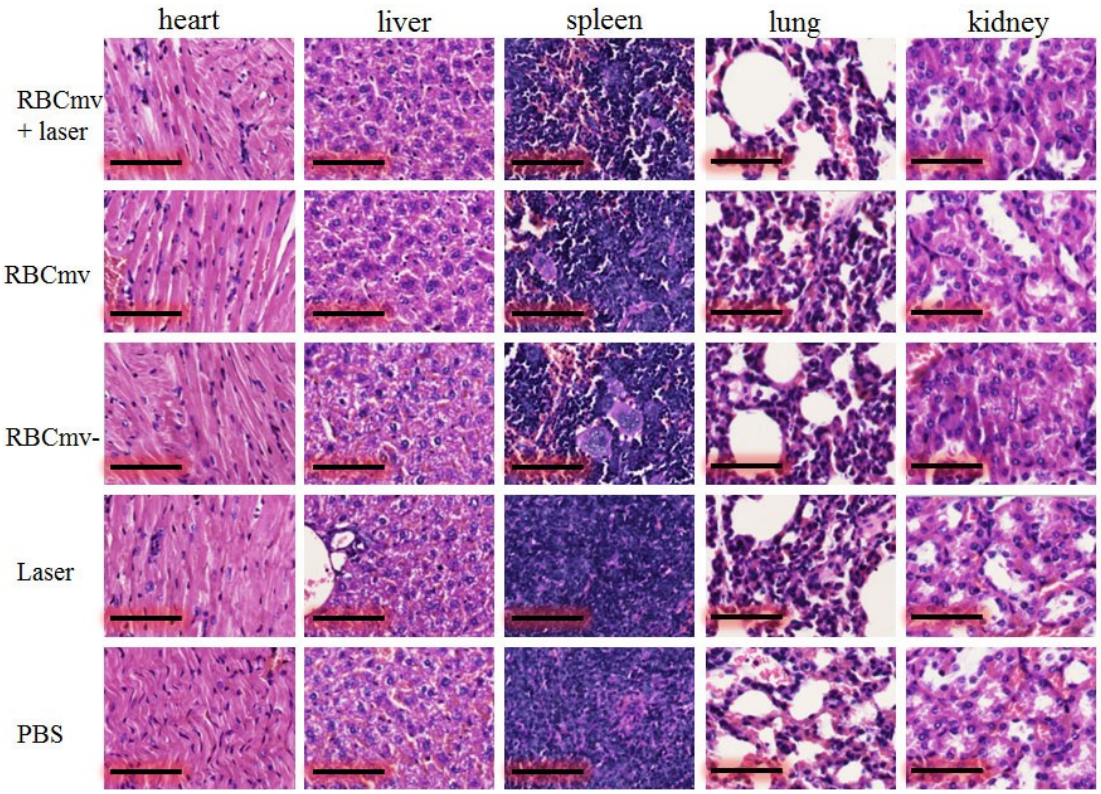


Fig. S13 H & E staining of organs (heart, liver, spleen, lung, and kidney) which were harvest after different treatments. Scale bars represent 200 μm.

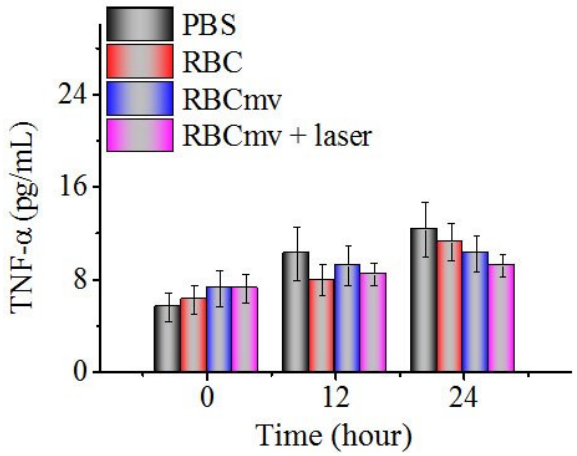


Figure S14 TNF-α level in blood after treatment of PBS, RBC, RBCmv and RBCmv + laser for 0 h, 12 h and 24 h post-injection.

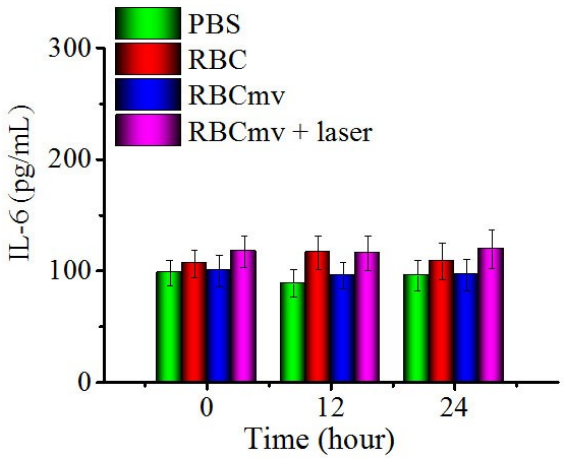


Figure S15 IL-6 level in blood after treatment of PBS, RBC, RBCmv and RBCmv + laser for 0 h, 12 h and 24 h post-injection.