Supporting Information

Fabrication of red blood cell membrane-camouflaged Cu_{2-x}Se nanoparticles for phototherapy in the second near-infrared window

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Experimental Section

Materials and instruments

Unless otherwise specified, all commercial reagents were used without further purification. CuSO$_4$·5H$_2$O, vitamin C (VC), Na$_2$SeO$_3$, sodium dodecyl sulfate aniline, 3,4-dihydroxybenzaldehyde, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichlorodihydro-fluorescein diacetate (DCFH-DA), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were purchased from Sigma. The live/dead cell imaging kit were purchased from Thermo Fisher Scientific.

The TEM images were captured with a Tecnai TMG2 F30, FEI operating. The zeta potentials and sizes were measured by the Zetasizer Nano ZS analyzer (Malvern Instruments). The UV-vis-NIR absorbance was measured on a Shimadzu Corporation UV-3600 spectrophotometer. The bio-TEM image was measured with a JEM100CX electron microscope. The 808 nm Fiber Laser and the NIR-II laser was produced by high power multimode pump laser (WaveParticle Technologyis). The temperature detection and thermal image record were measured on an infrared thermal imaging instrument (FLIR A325SC camera). Inductively coupled plasma mass spectrometry (ICP-MS) was carried out on a Thermo Scientific iCAP RQ series ICP-MS instrument. Electron spin resonance (ESR) spectra were using a Bruker Model A300 spectrometer.

Synthesis of Cu$_{2-x}$SeNPs

Cu$_{2-x}$Se nanoparticles (Cu$_{2-x}$SeNPs) were synthesized based on reported method, which were prepared by a one-step method in the room temperature. Typically, 1% sodium dodecyl sulfate solution (20 mL$^{-1}$) were added into beaker under vigorous stirring, followed by addition of 2 mL Na$_2$SeO$_3$ solution (0.1 M) and 4 mL VC (0.1 M). After 30 min and the color of solution turned red. Next, freshly prepared CuSO$_4$·5H$_2$O (5 mL, 0.1 M) solution was rapidly mixed with VC solution (5mL, 0.1 M). The color of the solution gradually changed from deep red to dark brown after reaction for 6 h at room temperature. The Cu$_{2-x}$SeNPs were purified by dialysis (MWCO 3.5kDa) for two days, and stored at 4 °C for further use. The concentration of Cu$_{2-x}$SeNPs were measured by using ICP-MS.

Preparation of RBC@Cu$_{2-x}$SeNPs

Cu$_{2-x}$SeNPs (0.4 mg·mL$^{-1}$) were prepared in water (5 mL), then the solution was filtered by 0.22 μm filter twice to remove large partials. RBC was obtained according to the previous literature’s method. The mixture of Cu$_{2-x}$SeNPs (5 mL) and RBC (2.5 mL) was extruded with the 100 nm membrane by a liposome
extrusion instrument (Avestin, Canada) to form RBC@Cu$_{2-x}$SeNPs.

**Deep-tissue photothermal and photodynamic properties**

*In vitro* photothermal properties of RBC@Cu$_{2-x}$SeNPs were evaluated by 1064 nm laser irradiation with different Cu$_{2-x}$Se concentrations (from 10 to 50 µg·mL$^{-1}$) at elevated NIR-II irradiation power density (0.8, 0.9, 1.0 W·cm$^{-2}$). To assess the tissue penetration *in vitro*, the 96-well plate were covered by chicken breast tissue with different thicknesses (0, 2, 4, 6, 8, and 10 mm), and followed by an 808 nm laser or a 1064 nm laser irradiation for 5 min at same concentration and laser power.

*In vitro* photodynamic properties of RBC@Cu$_{2-x}$SeNPs were evaluated by a typical DPBF assay. Briefly, 3 mL RBC@Cu$_{2-x}$SeNPs in the solution containing 10 µM DPBF was added into a cuvette, and irradiated with a NIR-II laser (1064 nm) at the power density of 0.8 W·cm$^{-2}$ for different time interval.

In order to further confirm the photodynamic properties of RBC@Cu$_{2-x}$SeNPs, electron spin resonance spectroscopic was used by a Bruker X-band A300 spectrometer at room temperature. The samples were placed into a quartz capillary tube. The spin trap 4-oxo-TEMP (2,2,6,6-Tetramethylpiperidine) was used to measure the production of singlet oxygen (\(^1\)O$_2$) under NIR irradiation (1064 nm, P = 0.8 W·cm$^{-2}$, t = 5 min).

**Calculation of the photothermal conversion efficiency**

The photothermal conversion efficiency of RBC@Cu$_{2-x}$SeNPs was measured according to the following equation.$^3$

\[
\eta = \frac{mc(T_{\text{max}} - T_{\text{sur}})}{I(1 - 10^{-A})\tau_s}
\]

Absorbance of RBC@Cu$_{2-x}$SeNPs at 1064 nm was confirmed by the identical concentration. And 1 mL RBC@Cu$_{2-x}$SeNPs of solution was prepared into a cuvette and irradiated with a 1064 nm laser, followed by natural cooling after the laser was turned off. In the current study, m is the solution mass and equal to 1.0 g, c stands for the heat capacity of water which equal to 4.2 J·g$^{-1}$, $T_{\text{max}}$ and $T_{\text{sur}}$, are the maximum temperatures of RBC@Cu$_{2-x}$SeNPs and water, respectively. $I$ represents the power density of laser, and $\tau_s$ is the system time constant which calculated according to the linear regression of the cooling profile.

**Cytotoxicity assay**

Human hepatocellular carcinoma (HepG2) cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin with 5% CO$_2$ at 37 °C.
(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was used to analyze the cytotoxicity of RBC@Cu$_{2-x}$SeNPs. Briefly, HepG2 cells were seeded in a 96-well plate. After 24 h incubation in an atmosphere of 5% CO$_2$ at 37 °C, RBC@Cu$_{2-x}$SeNPs of different concentrations were mixed with cells for 6 h. Then, 96-well plate was washed three times by phosphate-buffered saline (PBS). A 20 µL of MTT was added to 96-well plate to incubate for another 4 h. Finally, measuring the absorbance of each well at 490 nm using a microplate reader.

In vitro PTT and PDT in NIR-II window

Briefly, HepG2 cells were incubated in a 96-well plate, at 37 °C for 24 h. Then, one plate of wells were treated with 0.5 mM VC and then the media were replaced by RBC@Cu$_{2-x}$SeNPs at different Cu$_{2-x}$Se concentration (from 10 to 60 µg·mL$^{-1}$). After incubation for another 6 h, all of the wells were washed with PBS three times to remove the free RBC@Cu$_{2-x}$SeNPs. And then the two groups exposed to 1064 nm laser irradiation (0.8 W·cm$^{-2}$ and 5 min) at 4 °C and 37 °C. The groups were treated without laser irradiation as blank control. The photo toxicity was evaluated by the MTT as described above. All experiments were triplicated, and results were averaged.

Live-dead cell staining test

To visually confirm the anticancer activities of RBC@Cu$_{2-x}$SeNPs, HepG2 cells were co-incubated with propidium iodide (PI) and calcein-AM (AM) to distinguish the dead (red) cells from the live (green) cells. Only laser and RBC@Cu$_{2-x}$SeNPs in dark served as control groups and experimental groups (0.5 mM of VC) treated 6 h before RBC@Cu$_{2-x}$SeNPs addition at 37 °C, followed by exposed to 1064 nm laser irradiation at 4 °C and 37 °C (40 µg·mL$^{-1}$, 5 min and 0.8 W·cm$^{-2}$). Next, the cells were further incubated for another 24 h. Then, the plates were incubated with AM and PI for 30 min and washed by PBS for three times and the obtained cells were imaged by a fluorescence microscope.

Detection of ROS in vitro

To quantify the intracellular ROS of RBC@Cu$_{2-x}$SeNPs, 2′-7′-dichlorofluorescin diacetate (DCFH-DA) was be used, which could be change to non-fluorescent into fluorescent 2′-7′-dichlorofluorescin (DCF) when oxidized by ROS. Then, HepG2 cells were incubated for 24 h, and 40 µg·mL$^{-1}$ RBC@Cu$_{2-x}$SeNPs was added. Subsequently, 2µL non-fluorescent 2′,7′-dichlorofluorescin diacetate was added, after incubated for 6h, the solution was removed and washed with PBS three times. Followed exposed to 1064
nm laser irradiation for 5 min and incubated for another 30 min to detect ROS via CLSM.

**Cellular uptake and localization**

Cellular uptake was assessed via ICP-MS. HepG2 and RAW 264.7 cells were incubated in 10 cm plates for 24 h. Then the cells were incubated with RBC@Cu$_{2-x}$SeNPs (40 μg·mL$^{-1}$) with various times (2, 4, 6, 8, 10, 12 and 24 h). The free RBC@Cu$_{2-x}$SeNPs were washed with PBS three times. ICP-MS was used to evaluate the iron content in the harvested cells. The contrast cellular uptake experiments of RBC@Cu$_{2-x}$SeNPs and Cu$_{2-x}$SeNPs with HepG2 and RAW 264.7 as described above.

The intracellular localization of RBC@Cu$_{2-x}$SeNPs were directly observed by Bio-TEM. Typically, HepG2 cells were cultured with RBC@Cu$_{2-x}$SeNPs (the concentration of RBC@Cu$_{2-x}$SeNPs was 40 μg·mL$^{-1}$) for 6 and 12 h, respectively. When after varied co-incubation durations, the HepG2 cells were harvested and fixed for Bio-TEM characterization.

**Hemolysis assay**

Blood red cells were obtained by centrifugation, then washed and diluted with PBS. In general, blood samples (1.0 mL) were added into RBC@Cu$_{2-x}$SeNPs solution with concentrations 40 μg·mL$^{-1}$. PBS and deionized water were utilized as negative and positive controls, respectively. After being stationary at 37 °C for different time (0 to 24 h), the blood samples were centrifuged, and the obtained supernatant solution was used to measure their absorbance and then calculate the hemolysis ratio.

**Biodistribution and pharmacokinetics**

In pharmacokinetic experiments, HepG2 tumor-bearing mice were randomly divided into two groups and intravenously injected with free Cu$_{2-x}$SeNPs, RBC@Cu$_{2-x}$SeNPs (6.6 mg·kg$^{-1}$, Cu$_{2-x}$Se). A 20 μL blood was collected at different time intervals (10 min, 1, 4, 8, 12 and 24 h) after injection. The quantitative analysis of Cu element was measured by ICP-MS. And then, in vivo blood terminal half-life of Cu$_{2-x}$SeNPs and RBC@Cu$_{2-x}$SeNPs was assessed by a double component pharmacokinetic model. The biodistribution of Cu$_{2-x}$SeNPs and RBC@Cu$_{2-x}$SeNPs in tumor and other organs was evaluated in HepG2 tumor-bearing mice (n = 3). The mice were intravenously administered with Cu$_{2-x}$SeNPs and RBC@Cu$_{2-x}$SeNPs (6.6 mg·kg$^{-1}$) in PBS. Mice were sacrificed at predesignated time 24 h. Dissected organs and tumor were weighed and treated with concentrated nitric acid for a week, and then the concentration of Cu was evaluated by ICP-MS.
**In vivo phototherapy in NIR-II windows.**

All the Kunming strain mice used in this experiment were obtained from the Hospital of Jilin University. HepG2 cells were injected into the back of mice. After two weeks, the volume of tumors reach about 150 mm$^3$, and then, these tumor-bearing mice were randomly divided into seven groups (PBS, only laser, Cu$_{2-x}$SeNPs in dark, Cu$_{2-x}$SeNPs + Laser, RBC@Cu$_{2-x}$SeNPs in dark, RBC@Cu$_{2-x}$SeNPs + Laser). The RBC@Cu$_{2-x}$SeNPs at Cu$_{2-x}$Se concentration of 6.6 mg·kg$^{-1}$ were administered in intravenous group, after 4 h, the tumor sites were exposed to laser irradiation (1064 nm, 0.8 W cm$^{-2}$ and 5 min).

**Histological analysis.**

Mice from seven groups were sacrificed when experiments finished, and tissues (heart, liver, spleen, lung, kidney and tumor) of mice were collected, fixed with paraformaldehyde solution (4%, PBS), and then embedded by paraffin, finally utilized hematoxylin and eosin (H&E) to stain. The stained samples were observed by the microscope.

**Statistical analysis**

Data were expressed as mean ± standard deviation, and each experiment was evaluated significantly. Statistical analysis was performed by Student-Newmann-Keuls analysis of variance, and group data were analyzed by t test. Differences were considered significant at P< 0.05.

**References**


Supporting Figures

**Fig. S1** A) TEM images of Cu\textsubscript{2-x}SeNPs. B) Size distribution of Cu\textsubscript{2-x}SeNPs. C) Size distribution of RBC@Cu\textsubscript{2-x}SeNPs.
Fig. S2 A) The zeta potential of Cu$_{2-x}$SeNPs, RBC and RBC@Cu$_{2-x}$SeNPs. B) Size changes of RBC@Cu$_{2-x}$SeNPs in aqueous solution and DMEM containing with 10% FBS. C) The absorbance spectra of Cu$_{2-x}$SeNPs and RBC@Cu$_{2-x}$SeNPs at different concentrations.
Fig. S3  A) Temperature changes and corresponding photothermal heating pictures B) of water and RBC@Cu$_{2-x}$SeNPs at different concentrations under laser irradiation (1064 nm, 0.8·W·cm$^{-2}$). C) Temperature changes of RBC@Cu$_{2-x}$SeNPs at different power (1064 nm, 30 μg·mL$^{-1}$, 0.8, 0.9 and 1.0 W·cm$^{-2}$). D) Heating cycle curves of RBC@Cu$_{2-x}$SeNPs (1064 nm, 0.8W·cm$^{-2}$). E) Photothermal effect of RBC@Cu$_{2-x}$SeNPs for certain period (1064 nm, 0.8 W·cm$^{-2}$, 30 μg/mL), then turned off the laser. F) Compare the temperature changes of RBC@Cu$_{2-x}$SeNPs in 808 nm and 1064 nm laser irradiation at the various power density (0.6, 0.8 and 1.0 W·cm$^{-2}$, 30 μg·mL$^{-1}$).
Fig. S4 Temperature elevations of RBC@Cu$_{2-x}$SeNPs upon exposures to different tissue depth at 808 nm and 1064 nm laser irradiation (1.0 W·cm$^{-2}$, 40 μg·mL$^{-1}$).
Fig S5 Time-dependent $^1$O$_2$ generation of RBC@Cu$_{2-x}$SeNPs as irradiated by 1064 nm (0.8 W·cm$^{-2}$).
Fig. S6 A) Cellular uptake of RBC@Cu$_{2-x}$SeNPs at different treatment time in HepG2 cell. B) Macrophage uptake of Cu$_{2-x}$SeNPs and RBC@Cu$_{2-x}$SeNPs after 6 h of co-incubation. *P<0.05.
Fig. S7 Bio-TEM images of the localization of RBC@Cu$_{2-x}$SeNPs in HepG2 cells after 6 and 12 h treatment.
Fig. S8 The photographs of real-time heating temperature at various concentrations in the cells.
**Fig. S9** The quantitative analysis of hemolysis of the erythrocytes induced by Cu$_{2\times}$SeNPs and RBC@Cu$_{2\times}$SeNPs in erythrocyte dispersion at different times. B) Photographs of the erythrocytes induced by Cu$_{2\times}$SeNPs and RBC@Cu$_{2\times}$SeNPs in erythrocyte dispersion at 24 h.
**Fig. S10** Temperature elevations of tumor-bearing mice in Cu$_{2-x}$SeNPs and RBC@Cu$_{2-x}$SeNPs at the tumor location during 1064 nm laser irradiation. B) The IR thermal images of tumor-bearing mice in Cu$_{2-x}$SeNPs and RBC@Cu$_{2-x}$SeNPs at the tumor location. C) Body weight data of different groups after treatment in 20 days. D) H&E staining on tumor sites from HepG2 tumor-bearing mice after various treatments.
Fig. S11. H&E staining of the major organs (heart, liver, spleen, lung, and kidney) of HepG2 tumor-bearing mice after different treatments.