Supporting Information

Exploring the homing effect and enhanced drug delivery potential of small extracellular vesicles

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Contents

Experimental section

Materials and regents

Chloroauric acid ($HAuCl_4 \cdot 4H_2O$), sodium chloride (NaCl), sodium hydroxide (NaOH), trichloromethane ($CHCl_3$), dimethyl sulfoxide (C_2H_6SO, DMSO), 30% H_2O_2 and ascorbic acid (AA) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), polyvinylpyrrolidone (PVP, MW=10000), cupric acetate monohydrate ($Cu(AC)_2 \cdot H_2O$), sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$), cholesterol ($C_{27}H_{46}O$), doxorubicin hydrochloride ($C_{27}H_{29}NO_{11} \cdot HCl$, DOX), ferric chloride ($FeCl_3 \cdot 6H_2O$), tannic acid ($C_{76}H_{52}O_{46}$, TA), and 3,3',5,5'-tetramethylbenzidine ($C_{16}H_{20}N_2$, TMB) were purchased from Aladdin Biotechnology Co., Ltd. (Shanghai, China). Hydrogenated soybean phospholipids (HSPC) and 1-di-n-octadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI) were obtained from Macklin Biochemical Co., Ltd. (Shanghai, China). Rhodamine 6G (R6G) was obtained from Sigma-Aldrich (St. Louis, MO). Calcein-AM/PI Cell Viability/Cytotoxicity Assay Kit was obtained from Beyotime Biotechnology Co., Ltd. (Shanghai, China). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Yeasen Biotechnology Co., Ltd (Shanghai, China). Annexin V-FITC/PI apoptosis detection kit was obtained from Seven Biotechnology Co., Ltd. (Beijing, China). All reagents were used as received without additional purification. Deionized water (DI water) was used throughout the experiments.

Synthesis of Au NPs seed solution

Au NPs seeds were synthesized based on a previously reported work.[1] First, $HAuCl_4 \cdot 4H_2O$ solution (25 mmol/L, 0.5 mL) was dissolved in 50 mL DI water in a 250 mL flask. After boiling continuously for 5 min at 120℃, sodium citrate solution (38 mmol/L, 1.2 mL) was rapidly added and stirred for another 30 min. After cooling to room temperature, the solution was stored at room temperature for further use.

Synthesis of Au@CuS NPs

The synthesis of Au@CuS NPs is similar to a previously reported work.[1] Briefly, PVP solution (8 mmol/L, 7.5 mL) was added dropwise to the above synthesized Au NPs seed solution and stirred for 30 min at 70℃. Then, $Cu(AC)_2 \cdot H_2O$ solution (0.1 mol/L, 562.5 μL) was added drop by drop under stirring. The NaOH solution (1 mol/L, 525 μL) was then added. After continuous stirring, AA solution (0.1 mol/L, 3.375 mL) was added rapidly, and the color of the solution turned blue. Subsequently, the solution reacted for 30 min at room temperature and then was transferred to a 90℃-oil bath. Then, $Na_2S \cdot 9H_2O$ solution (0.1 mol/L, 1.687 mL)

was added and stirred for 2 h. Au@CuS NPs were successfully synthesized, washed with DI water for three times, and stored at -4℃ for further use.

Photothermal performance of Au@CuS NPs

To evaluate the photothermal performance of the prepared Au@CuS NPs, an 808 nm laser was chosen to irradiate the Au@CuS NPs solutions with different concentrations (12.5, 25, 50, 100, and 200 μg/mL, 0.2 mL) and power densities (0.5, 1, 1.5, and 2 W/cm^2) for 6 min. In addition, to investigate the photothermal stability of Au@CuS NPs, 25 μg/mL of Au@CuS NPs solution was irradiated with the 808 nm laser (2 W/cm^2) for 6 min (laser on), followed by cooling to room temperature without irradiation (laser off). This process was repeated five cycles, and the temperature was monitored.

Calculation of the photothermal conversion efficiency

The photothermal conversion efficiency (η) of Au@CuS NPs was measured. The solutions of 200 μg/mL Au@CuS NPs were irradiated with the laser (808 nm, 2 W/cm^2) for 6 min, and then the laser was turned off to cool them to room temperature. The photothermal conversion efficiency was calculated using the method described in a previous study.[2, 3]

$$\eta = \frac{hA(T_{max} - T_{amb}) - hA(T_{max,water} - T_{amb})}{I(1 - 10^{-A_\lambda})} \quad (1)$$

$$hA = \frac{m_0 c_0}{\tau_s} \quad (2)$$

where T_{amb} is the surrounding temperature, T_{max} and $T_{max,\,water}$ are the maximum temperature of Au@CuS NPs aqueous solution and DI water, respectively; I is the laser power; A_λ is the absorbance of the sample under the irradiation of an 808 nm laser; h is the heat transfer coefficient; A is the surface area of the container; m_0 is the weight of the aqueous solution; c_0 is the specific heat capacity of water; and τ_s is the time constant, which can be calculated by introducing θ as the ratio of $(T - T_{amb})/(T_{max} - T_{amb})$, where T is the instant temperature. τ_s then can be obtained from the slope of the $-\ln(\theta)$ –time plot.

Peroxide-like catalytic activity of Au@CuS NPs

The peroxidase-like catalytic activity of Au@CuS NPs was investigated using TMB as substrate. Specifically, 80 μL of TMB (25 mmol/L), 10 μL of H_2O_2 (10 mmol/L), and 100 μL of HAc-NaAc (pH = 4.19) were successively added to 10 μL of Au@CuS NPs solution (200 μg/mL). Meanwhile, the control groups were set up as follows: (i) TMB, (ii) TMB + Au@CuS NPs, (iii) TMB + H_2O_2, and (iv) Au@CuS NPs + H_2O_2. DI water was added to make the final concentration of each component consistent. Subsequently, the absorbance of all the reaction

solutions at 652 nm was measured by using a multimode microplate reader (Biotek, Synergy H1).

To assess the impact of the photothermal effect of Au@CuS NPs on the peroxidase's catalytic activity, TMB + Au@CuS NPs + H_2O_2 was incubated at 10℃, 30℃, 50℃, and 70℃, respectively. After that, the absorbance of all the reaction solutions at 652 nm was measured using the multimode microplate reader. Au@CuS NPs solutions with different concentrations were used to verify the effect of concentration on the catalytic activity of peroxidase. Subsequently, the absorbance of all the reaction solutions at 652 nm was measured.

Isolation of sEVs

To isolate HeLa sEVs, HeLa cells were cultured in fresh DMEM medium at 37℃ under 5% CO_2. After 48 h of culture, the cell culture supernatant was collected. sEVs were collected by ultrafast centrifugation. Briefly, the medium was centrifuged at 300×g for 10 min to remove cells, and then at 2000×g for 10 min to remove dead cells, after which the supernatant was centrifuged at 10000 rcf at 4℃ for 30 min. The obtained supernatant was filtered with 0.22 μm filter membrane. Subsequently, the filtered supernatant was ultracentrifuged at 150000×g at 4℃ for 3 h. The supernatant was aspirated, and the superionization tube was repeatedly blown to obtain sEVs.[4, 5] The isolation of sEVs derived from MCF-7 cells and LoVo cells is consistent with the previous procedures.

Synthesis of Lip-Au@CuS NPs/DOX

Lip-Au@CuS NPs/DOX were prepared using a previously reported thin-film hydration method.[6] Briefly, hydrogenated soybean phospholipids (10 mg) and cholesterol (2.04 mg) were dissolved in 6 mL of chloroform, and then the solvent was evaporated at 35℃ for 50 min to form a film. Thereafter, Au@CuS NPs and DOX were added and shaken for 2.5 h on a multi-purpose decolorization shaker. The solution was hydrated in a 45℃-water bath for 1 h and then centrifuged to remove any unwrapped nanoparticles. The resulting solution was extruded 15 times through a polycarbonate membrane with 200 nm pores to obtain liposomes of uniform size.

Preparation and characterization of sEV-(Lip-Au@CuS NPs/DOX)

Lip-Au@CuS NPs/DOX coated with TA was prepared based on the coordination reaction of metal ions and polyphenol.[7] Generally, $FeCl_3 \cdot 6H_2O$ (1.2 mg/mL) and TA (6 mg/mL) were added to the Lip-Au@CuS NPs/DOX successively. Then MOPS buffer was added, and the mixture was vortexed for 30 min, followed by centrifugation and suspension in deionized

water. (Lip-Au@CuS NPs/DOX)-TA was thus synthesized and stored at 4℃ for further use. Subsequently, sEVs at a concentration of 4.3×10^8 particles/mL and (Lip-Au@CuS NPs/DOX)-TA reacted at 4℃ for 4 h to assemble sEV-(Lip-Au@CuS NPs/DOX).

The successful assembly of sEV-(Lip-Au@CuS NPs/DOX) was validated through a fluorescence co-localization assay. sEVs were resuspended in 1 mL PBS containing 10 μL of DiO (1 mM), and Lip-Au@CuS NPs/DOX was resuspended in 1 mL PBS containing 10 μL of DiI (1 mM). Then, they were incubated at 37℃ for 50 min and filtered through a 100 kDa ultrafiltration to remove excess dyes, followed by confocal laser scanning microscope (CLSM) detection (Olympus, FV1200).

Detection of reactive oxygen species (ROS) *in vitro*

2', 7'-dichlorofluorescein diacetate (DCFH-DA) was used to detect intracellular ROS in HeLa cells. HeLa cells were seeded in confocal dishes and cultured for 24 h. After that, different concentrations of sEV-(Lip-Au@CuS NPs/DOX) were added and incubated for 4 h. After the cells were washed with PBS, DCFH-DA (8 μmol/L) was added and incubated for 30 min. Then, intracellular ROS was imaged using a confocal laser scanning microscope (CLSM, Olympus, FV1200).

Cellular uptake of sEV-(Lip-Au@CuS NPs/DOX)

Cy5 was used to label sEV through a substitution reaction according to the reported work.[8] First, periodate was used to oxidize the hydroxyl groups on the sEV surface. Then, Cy5-hydrazide (50 μM) was added and incubated for 2 h to obtain Cy5-labeled sEV. Rhodamine 6G (R6G) was used to label Lip-Au@CuS NPs/DOX by interacting with the benzene ring structure of TA. Briefly, R6G (100 μM) was added after forming the TA film to obtain R6G-labeled Lip-Au@CuS NPs/DOX. Cells were seeded in confocal dishes and cultured for 24 h. Then, fresh culture medium containing sEV-(Lip-Au@CuS NPs/DOX) was added and incubated at 37℃ for 2 h. Finally, the cells were washed with PBS for three times and observed by CLSM.

***In vitro* cytotoxicity and apoptosis assay of sEV-(Lip-Au@CuS NPs/DOX)**

MTT assay was used to verify the biocompatibility of sEV-(Lip-Au@CuS NPs/DOX) to HeLa cells. Briefly, HeLa cells were seeded into 96-well plates and cultured overnight. DMEM medium containing sEV-(Lip-Au@CuS NPs/DOX) with different concentrations of Au@CuS NPs (5, 10, 25, 50, 100, 150, and 200 μg/mL) and 100 μg/mL of DOX was added. After

incubated for 24 h, the cells were washed with PBS. Then, DMEM medium containing 5% MTT was added to each well and incubated for 4 h. Afterward, the medium was removed, and DMSO (120 μL) was added to dissolve the insoluble crystals of formazan formed by living cells. After further incubation at 37℃ for 30 min, the absorbance at 570 nm was measured. Each group was replicated three times.

Flow cytometry assay was used to evaluate the synergistic therapeutic effect of sEV-(Lip-Au@CuS NPs/DOX). HeLa cells were seeded in 35 mm dishes and cultured for 24 h. The cells were then treated with various nanocarriers and divided into laser groups and non-laser groups. For the non-laser groups, the cells were incubated with PBS, 200 μg/mL of sEV-(Lip-Au@CuS NPs), 100 μg/mL of sEV-(Lip-DOX), and sEV-(Lip-Au@CuS NPs/DOX) containing 200 μg/mL of Au@CuS NPs and 100 μg/mL of DOX. For the laser groups, the cells were incubated with PBS, 200 μg/mL of sEV-(Lip-Au@CuS NPs), 100 μg/mL of sEV-(Lip-DOX), and sEV-(Lip-Au@CuS NPs/DOX) containing 200 μg/mL of Au@CuS NPs and 100 μg/mL of DOX. After 4 h of coculture, the cells were washed with PBS. The laser groups were irradiated with the 808 nm laser for 6 min (2 W/cm^2), and the non-laser groups were kept in dark. After the incubation of the cells with nanocarriers for 4 h, they were washed three times with PBS. Next, the cells were digested and collected using trypsin without EDTA. All the cells were washed with PBS and 5 μL of Annexin V-FITC and 5 μL of PI solutions were added and allowed to react for 15 min for the flow cytometry test (BD Accuri C6 Plus).

For the calcein-AM/PI co-staining assay, the cell culture step is the same as that for the flow cytometry assay. After the incubation of the cells with nanocarriers for 4 h, they were washed three times with PBS. The cells were then divided into laser groups and non-laser groups. The cells in the laser groups were irradiated with the 808 nm laser (2 W/cm^2) for 6 min. Then, calcein-AM/PI co-staining reagents were added and incubated for 30 min. The cells were observed using a fluorescence microscope (Olympus, BX53M).

Statistical analysis

The quantitative data were analyzed using an independent sample *t* test. The *P* value represents the statistical significance of differences among the groups. The statistical significance levels are denoted as follows: ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

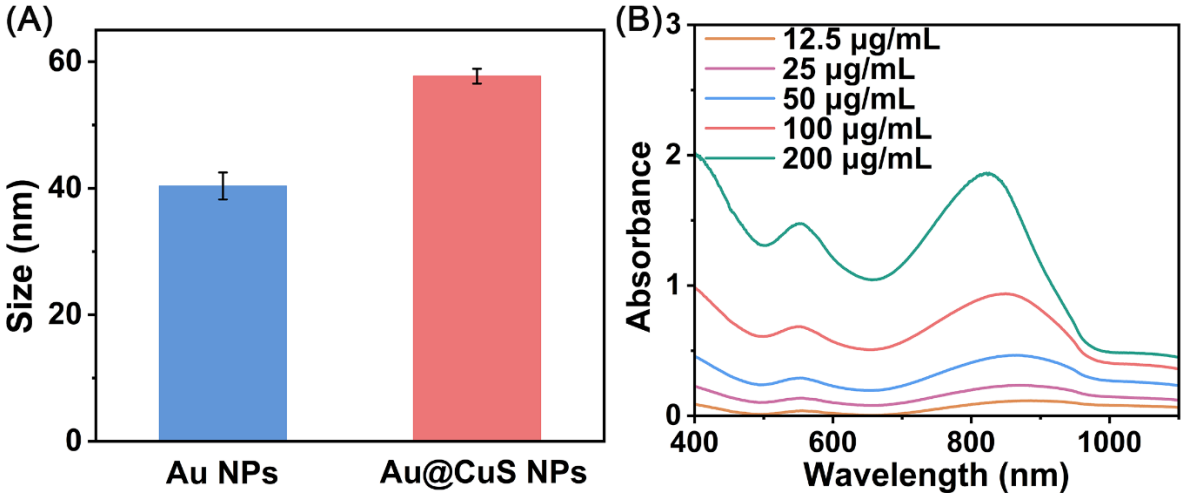

Figure S1. (A) Hydrodynamic diameters of Au NPs and Au@CuS NPs (n = 3). (B) UV-vis absorption spectra of Au@CuS NPs at different concentrations.

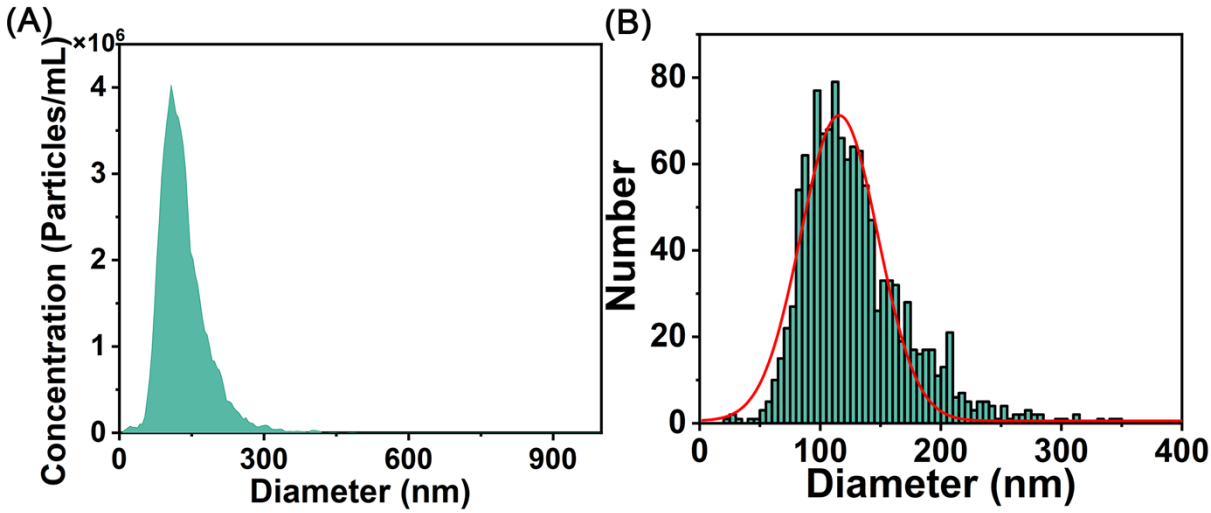

Figure S2. Concentration (A) and size distribution (B) of HeLa cell-derived sEVs measured by NTA (n = 3).

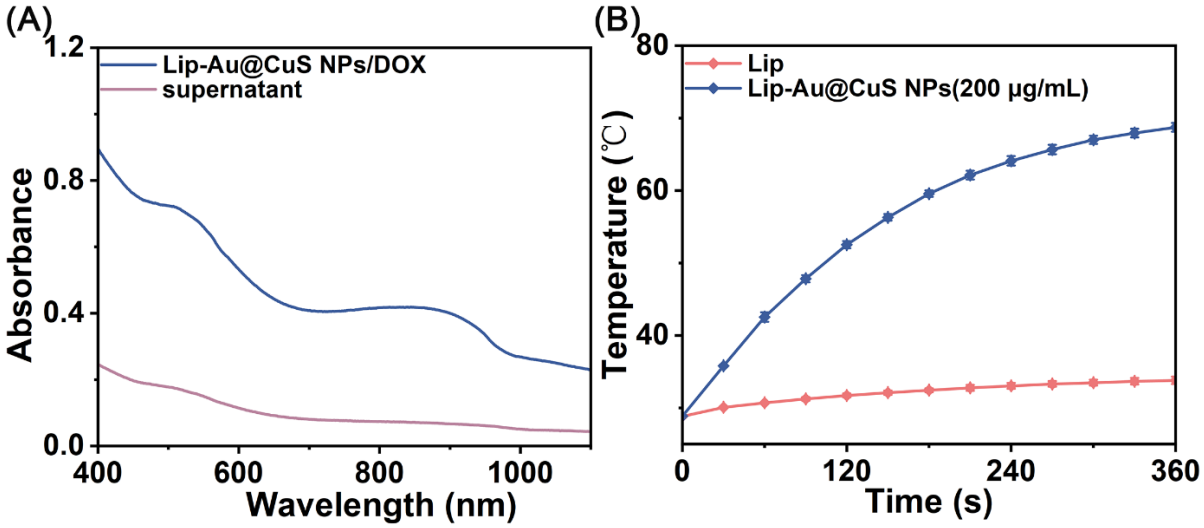

Figure S3. (A) UV-vis absorption spectra of Lip-Au@CuS NPs/DOX and supernatant. (B) Photothermal performance of Lip-Au@CuS NPs (200 μg/mL) under 808 nm laser irradiation (2 W/cm^2) (n = 3).

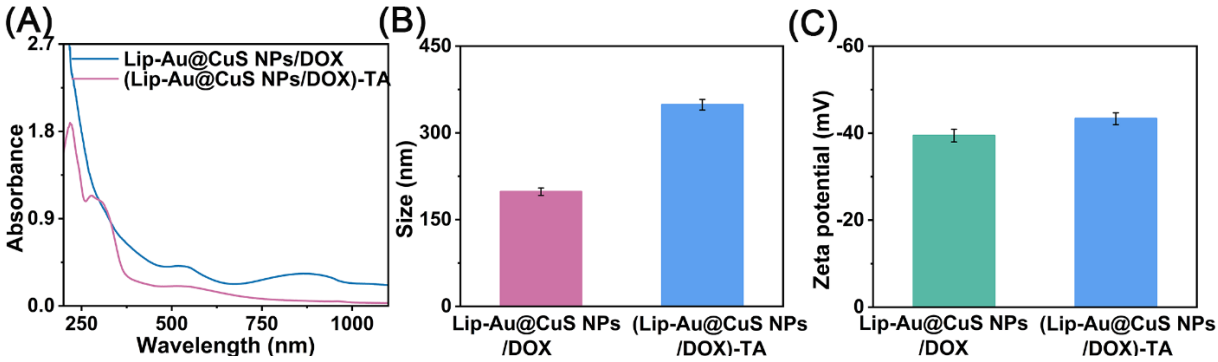

Figure S4. UV-vis absorption spectra (A), hydrodynamic diameters (B), and zeta potentials (C) of Lip-Au@CuS NPs/DOX and (Lip-Au@CuS NPs/DOX)-TA (n = 3).

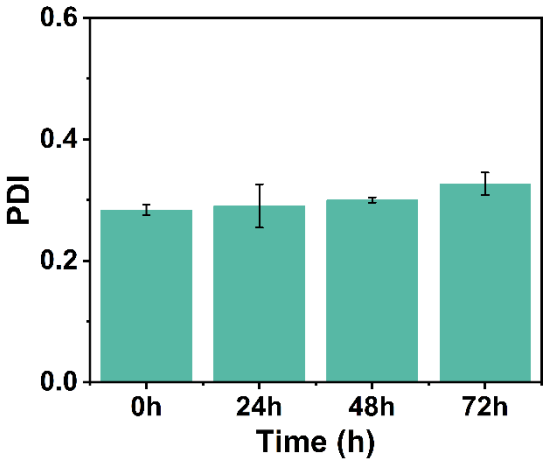

Figure S5. The polydispersity indexes (PDIs) of sEV-(Lip-Au@CuS NPs/DOX) in PBS under flow condition (n = 3).

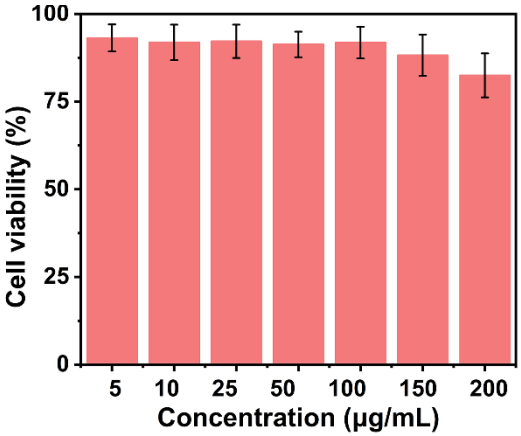

Figure S6. Cell viability of HeLa cells after incubation with various concentrations of sEVs-(Lip-Au@CuS NPs/DOX) for 24 h (n = 3).

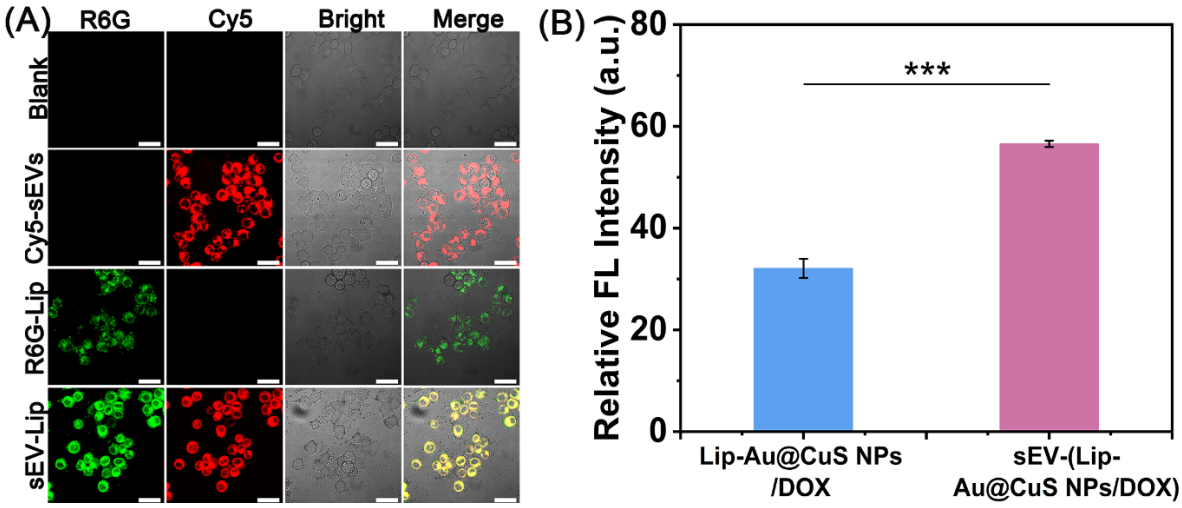

Figure S7. (A) CLSM images of HeLa cells after incubating with Cy5-labeled sEV, R6G-labeled Lip-Au@CuS NPs/DOX, and sEV (Cy5)-(Lip-Au@CuS NPs/DOX) (R6G). Scale bar: 40 μm. (B) Relative fluorescence intensities of Lip-Au@CuS NPs/DOX and sEV-(Lip-Au@CuS NPs/DOX) respectively endocytosed by HeLa cells. ***p<0.001, (n = 5).

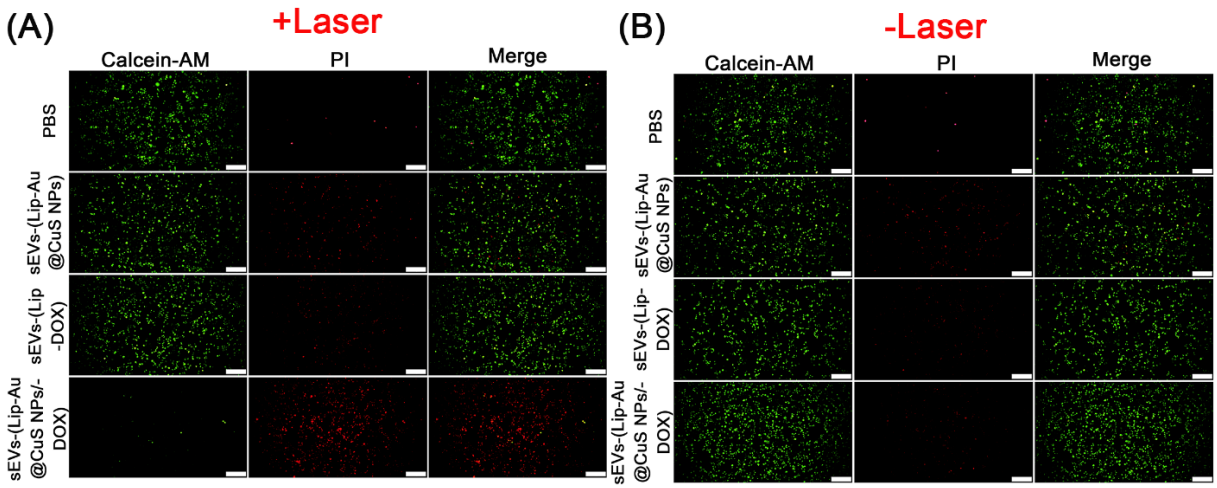

Figure S8. Calcein-AM and PI co-staining of HeLa cells after incubating with PBS and different dimers with (A) or without (B) 808 nm laser irradiation (2 W/cm^2). Green: live cells, Red: dead cells. Scale bar: 200 nm (n = 3).

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