Supplementary Information

Synergetic ER-targeting Liposome Nanocarriers with Anti-phagocytic property for Enhanced Tumors Theranostics

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1. Materials.

1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly (ethylene glycol)$_{2000}$]-maleimide (DSPE-PEG-MAL) was purchased from Nanosoft Biotechnology LLC (USA). Soybean phosphatidylcholine (SPC) and cholesterol (CHO) were purchased from A.V.T. Pharmaceutical Co., Ltd. Doxorubicin Hydrochloride (DOX-HCl) was purchased from Beijing Huatai Chemical Materials Corporation (China). 9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acid. Wang resin was purchased from GL Biochem (China). All chemicals used in this work were of analytical grade and were used as received.

Thiazolyl blue tetrazolium bromide (MTT), trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), fluorescein isothiocyanate (FITC) and Hoechst 33342 were purchased from Sigma-Aldrich. 4-Methoxybenzophenone was purchased from Alfa Aesar (China). Triisopropylsilane (Tips) was purchased from Acros Organics. 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (Dir) were obtained from Sigma-Aldrich (USA). BCA protein assay kit was purchased from Beyotime Biotechnology (China). Poly (vinylidene difluoride) membranes were purchased from Merck Millipore (Germany). Dulbecco's modified Eagle's medium (DMEM)/high-glucose medium were purchased from GE Healthcare Life Sciences. The human breast cancer cell line MCF-7 and macrophage cell line RAW 264.7 were purchased from the Cell Resource Center, Chinese Academy of Medical Sciences (China). All cells were supplemented with 10% fetal bovine serum (Gibco) and 100 U/mL penicillin and 100 U/mL streptomycin (Gibco). All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 ºC. Female BALB/c nude mice (6-8-week-old) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China).

2. Synthesis of self-peptide (SP).

Peptides were synthesized by solid-phase methods using a standard Fmoc-Chemistry. Wang Resin (Rapp Polymere, Germany, loading 0.34 mmol/g) is used as the solid

phase support. All the synthesis process of peptides was carried out in dehydrous DMF. Qualitative Fmoc deprotection was confirmed by a ninhydrin test (ninhydrin, phenol, Vitamin C 1:1:1 v/v). During the coupling step, the HBTU (4 mM) and Fmoc-amino acid (4 mM) were dissolved in DMF containing NMM (0.4 mM). Cleavage from the resin and deprotection of the amino acid side chains were performed by reaction with the mixture of TFA (95% v/v), H_2O (2.5% v/v) and TIPS (2.5% v/v) for 30 min in ice bath, then at room temperature for another 3 h. After separated from the resin, the mixture above was vacuum-rotary-evaporated to remove TFA. The crude peptides were then precipitated in cold anhydrous diethyl ether, collected by centrifuge and dried under vacuum. The purified peptides were concentrated by a vacuum centrifuge concentrator with speed 1500 rpm (Jiaimu CV200, China), Cold trap temperature with -50 °C (Jiaimu JM50, China). Finally, the peptides were purified by preparative reversed-phase high performance liquid chromatography (HPLC). The purified peptide was determined by MALDI-TOF mass spectrometry (Bruker Daltonics, Germany).

3. Conjugation between peptide/anti-ER and DSPE-PEG-MAL.

SP-DSPE-PEG was synthesized by conjugation of self-peptide with DSPE-PEG-MAL. Briefly, SP and DSPE-PEG-MAL (1:1, w/w) were dissolved in deionized water, stirring at room temperature for 24 h. Then the reaction mixture was purified by the dialysis bag with a molecular weight cut-off of 3,500 Da for 24 h and then concentrated by a vacuum centrifuge concentrator with speed 1500 rpm (Jiaimu CV200, China), Cold trap temperature with -50 °C (Jiaimu JM50, China). The coupling process was monitored by HPLC of which the peak of peptide was decreased along with the time. The product was analyzed by MALDI-TOF-MS using a Microflex LRF System spectrometer (Bruker Daltonics, USA). The anti-ER-DSPE-PEG was synthesized in the same manner as above, anti-ER and DSPE-PEG-COOH (molar ratio = 1:1) were dissolved in deionized water, stirring at room temperature for 24 h continually. A waters (e2695) HPLC system was employed to detect the conjugation product between DSPE-PEG and anti-ER antibody. We used

GSK gel-chromatography column (TOSOH Co. TSK-GEL G3000SW). The mobile phase is 20 nM Phosphate Buffer (pH 6.8) and 200 nM NaCl. The product was separated under the isocratic elution with the flow rate of 1 mL/min. The detection wavelength is 280 nm.

4. Preparation and optimization of DOX encapsulated liposomes.

DOX encapsulated functionalized liposome were prepared based on a film dispersion method. DOX was dissolved in 1 mL methanol at room temperature, mixed with soy phosphatidylcholine (SPC), cholesterol (CHO), SP-DSPE-PEG, anti-ER-DSPE-PEG and DOX in 5 mL dichloromethane/methanol (volume ratio 2:1). The ratio among SPC, CHO, SP-DSPE-PEG, anti-ER-DSPE-PEG and DOX (w/w/w/w/w) was set in four values to find the optimized conditions. i.e. 16:4:4:1:2, 8:2:2:1:1, 16:4:4:3:2 and 8:2:2:2:1. The mixture solvent was removed by vacuum rotary evaporation to form a dry drug-containing lipid film. The dried film was hydrated with 5 ml phosphate-buffered saline (PBS) at 60 °C for 15 min. The final product was dialyzed by a dialysis bag with the molecule weight cut-off of 5kDa. Finally, the liposome suspension was eluted by 0.22 μm hyperfiltration membrane.

5. Size and Zeta Potential Analysis and Transmission Electron Microscopy of the prepared liposomes.

Particle size (hydrous diameter) and zeta potential of nanoparticle dispersions ($SELS^{DOX}$ and LS^{DOX}) were measured at room temperature by dynamic light scattering (DLS) using a Zetasizer 5000 analyzer (Malvern Instruments, Malvern, Worcestershire, U.K.). To obtain detailed structural and morphological information, $SELS^{DOX}$ and LS^{DOX} were imaged using Transmission Electron Microscope (HT7700 TEM, Japan) with 200 kV acceleration voltage. The process was described as following: drop 6 μL liposome suspension onto carbon membrane supported copper mesh for 2 minutes and then moved away the residual liposome suspension. When the membrane was dried, 6 μL uranyl acetate (1%) was added to the membrane for 3 minutes. Then, remove the staining solution and dry the membrane at room temperature. Finally, the samples were observed by TEM.

6. Encapsulation efficiency detection of the liposomes.

Weighed soy phospholipid (4 mg) and cholesterol (1 mg) are dissolved in an organic solvent mixed with dichloromethane and methanol (2:1, v/v). Then, they are slowly rotated and evaporated with a gradient pressure (condition: 1) 0.01 pa for 30 min, 2) 0.02 pa for 10 min, 3) 0.03 pa for 10 min, 4) 0.06 pa for 10 min and 5) vacuum for 10 min). The organic solvent was removed to form a uniform phospholipid film on the wall of the bottle. Then, an appropriate amount of PBS was added. Hydration was performed in a 60 ℃ water bath to curl the phospholipid membrane into bilayer vesicles. Finally, a uniformly dispersed spherical liposome was formed by ultrasonic dispersion. The standard curve of DOX concentration was measured with a series of dilutions: 22, 20, 18, 16, 14, 12, 10, 8, 6 and 4 μg/mL (DOX dissolved in PBS). Fluorescence intensities of these solutions were measured (ex: 480 nm; em: 560-590 nm) to obtain a standard curve. It shows a linear standard curve with R^2 of 0.99832. The liposome encapsulation efficiency was calculated from the amount of drug using the centrifugation technique. Briefly, total doxorubicin was determined after having dissolved and un-loaded DOX was centrifuged by ultrafiltration centrifugal tube at 50000 rpm for 20 min. The amount of unloaded DOX in the outer tube was determined by measuring the fluorescence intensity. The total amount of DOX in the system is sum of the amount of DOX loaded in each liposome ($W_{encapsulation}$) and unloaded DOX ($W_{unloaded}$). Encapsulation efficiency (EE) was calculated according to the formula:

$$EE = (W_{encapsulation} / W_{total}) \times 100\%$$

7. Western blot assay for cheking the ER expression in MCF-7 cells.

MCF-7 cells were cultured in the medium at the density of 10^7 per dish. Cells were washed twice with ice-cold PBS and lysed in lysis buffer (Sigma, USA) on ice for 30 min. Cell lysates were collected by centrifugation at 4 ℃ for 30 min, and the total protein was measured using the BCA protein assay kit (Beyotime Biotechnology, China). According to the standard western blot procedures, proteins were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Merck

Millipore, DE). The membranes were then blocked for 1 h in 5% non-fat milk in PBST and incubated with anti-ER (Abcam, 1:500) antibody and β-actin antibody (Biosynthesis, 1:1000) respectively overnight at 4 ℃. After washing 3 times with PBST for 10 min each, the membranes were incubated with horseradish peroxidase-coupled isotype-specific secondary antibodies for 1 h at room temperature. Then, the membranes were washed 3 times with PBST for 10 min each. The immune complexes were detected by ChemiDoc MP imaging system (Bio-Rad, USA).

8. Detection for cellular uptake of DOX-loaded liposomes by flow cytometry.

The cellular uptake of DOX-loaded liposomes was detected by flow cytometry. Generally, MCF-7 cells were seeded into six-well plates at a density of 5×10^5 cells/well and cultured at 37 °C for 24 h. Cells were washed twice with PBS (pH 7.4) to remove the remnant growth medium, and then incubated in serum-free medium containing various DOX formulations at the final DOX concentration of 20 μg/mL. After 30 min incubation, the cells were washed twice with cold PBS, then re-suspended in 500 mL of PBS. We explored the cellular uptake of $SELS^{DOX}$, ELS^{DOX}, SLS^{DOX} and LS^{DOX} by MCF-7 cells using flow cytometry (BD Accuri C6). We use FlowJo software to analyze the related data. We selected the cells incubated with PBS as control for creating gates. Gating is the process of subsetting collected events for further analysis. This allows us to control each sample's gates and choose to re-batch the gates of the whole set of samples. The number of cells in the gates is 10,000. We selected the fluorescence detection channel as FL2 (DOX Ex: 488 nm, Em: 560-590 nm). The purpose is to detect the corresponding fluorescent intensity of all the groups of samples.

9. Drug release and cytotoxicity assay *in Vitro.*

1mg of DOX-loaded targeted or non-targeted liposomes were dispersed uniformly in 4 ml of medium and then placed in a dialysis bag with a molecular weight cut-off of 3500 Da. We immersed the dialysis bag in 40 ml of PBS (pH 7.4) containing 1% serum in a 50 ml beaker and kept at 37 °C under a horizontal laboratory shaker at 200 rpm. The release medium was removed and refreshed ever two hours during the

whole 48- hour release process. The amount of released DOX was analyzed by a UV-Vis-NIR spectrophotometer at Ex 485 nm/Em 590 nm.

MCF-7 cells (150 μL) were seeded into each well of a 96-well plate at a density of 5 $\times 10^3$ cells/well, and allowed to adhere for 24 h at 37 °C. The growth medium was replaced with 150 μL of fresh medium containing $SELS^{DOX}$, ELS^{DOX}, SLS^{DOX}, LS^{DOX} and free DOX for 24 h at different doxorubicin concentrations ranging from 0.001 to 100 μg/mL. Subsequently, the medium was replaced with 100 μL 5 mg/mL MTT for additional 4 h at 37 °C. After that, the growth medium was removed, and 150 μL DMSO solution was added to each well to ensure solubilization of formazan crystals. The absorbance was recorded at a wavelength of 570 nm using an automated M200 microplate reader (Tecan, Durham, USA). Untreated cells were used as control. The cell viability was expressed as percentage calculated with the absorbance obtained from control well without drug treatment. Each concentration gradient was carried out with three replicates.

10. Confocal fluorescence imaging of MCF-7.

Human breast cancer cell line MCF-7 were seeded (1×10^5 mL/cells) into 35 mm microscope dishes and cultured overnight for the adhesion of cells. DOX was dissolved in 1 mL methanol at room temperature, mixed with soy phosphatidylcholine (SPC), cholesterol (CHO), SP-DSPE-PEG, anti-ER-DSPE-PEG and DOX in 5 mL dichloromethane/methanol (volume ratio 2:1). The ratio among SPC, CHO, SP-DSPE-PEG, anti-ER-DSPE-PEG and DOX (w/w/w/w/w) were set in four values to find the optimized conditions. i.e. 16:4:4:1:2, 8:2:2:1:1, 16:4:4:3:2 and 8:2:2:2:1. When the ratio is 8:2:2:2:1, $SELS^{DOX}$ shows the best drug delivery efficiency. $SELS^{DOX}$, ELS^{DOX}, SLS^{DOX} and LS^{DOX} were dissolved in cell culture medium at the concentration of 20 μg/mL. The cells were then washed three times with cold PBS and incubated with Hoechst 33342 (10 μg/mL, 200 μL) for at 37 °C for 15 min. Afterwards, cells were incubated with $SELS^{DOX}$, ELS^{DOX}, SLS^{DOX} or LS^{DOX} at 4 °C for 30 min, 1 h, 2 h and 4 h, respectively. Finally, the cells were washed three times with cold PBS. Confocal fluorescence imaging was performed on an Olympus

FV1000-IX81 confocal-laser scanning microscope (CLSM). The objective lens used for imaging was a UPLSAPO 63×oil-immersion objective (Olympus).

11. Phagocytosis of the liposomes by macrophages.

Cellular phagocytosis of $SELS^{DOX}$, ELS^{DOX}, SLS^{DOX} and LS^{DOX} at different time was investigated by CLSM observation. Macrophages Raw 264.7 were incubated in 35 mm microscope dishes and cultured overnight at 37 °C for 24 h. Cells were incubated with Hoechst 33342 (10 μg/mL, 200 μL) for 15 min, then cells were incubated with $SELS^{DOX}$, ELS^{DOX}, SLS^{DOX} or LS^{DOX} (20 μg/mL, 200 μL) for 3 h, 6 h, 9 h, 12 h at 37 °C. Macrophages were washed with PBS and fluorescence microscopy by Olympus IX81 confocal laser scanning microscope. The total number of particles associated to macrophages were detected according to the cell fluorescent intensity.

12. *In vivo* biodistribution imaging of $SELS^{Dir}$.

Female BALB/c nude mice (6 week) with the body weight of ~20 g was purchased from *Vital River Laboratory Animal Center (Beijing, China)* and kept under specific aseptic conditions with free access to standard food and water. All the animal experiments were conducted in compliance with the guide for the care and use of laboratory animals of *Beijing University Animal Study Committee's requirements*. The xenograft tumors were established by subcutaneously injection of 1×10^7/mL MCF-7 cells (150 μL) to the right hind leg of mice. Tumor growth was measured periodically and until the tumors reached to grow to ~5 mm in diameter. 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbonyaineiodide (Dir, Invitrogen) with emission wavelength maxima centered at 748 nm. Mice were injected via the caudal vein with $SELS^{Dir}$, ELS^{Dir}, SLS^{Dir}, LS^{Dir} (200 μL) at a dose of 1 μg/ml Dir. PBS was served as control. Near-infrared imaging was carried out after 2h, 4 h, 6 h, 8 h, 10 h, and 24 h by using Maestro *in vivo* spectrum imaging system (Cambridge Research & Instrumentation, Woburn, MA). Next, organs were excised for *ex vivo* fluorescence imaging. All images were taken with a ten-second exposure time to ensure consistency in the data.

Normal BALB/c nude mice (n = 3) were intravenously injected with $SELS^{Dir}$, ELS^{Dir},

SLS^{Dir}, LS^{Dir} and free Dir (200 μL, 1 μg/mL Dir equivalent), respectively. At each time point after injection of the material, 100 μl of blood was taken from the tail vein of nude mice for half-life detection. Blood is added in the EDTA anticoagulation tube. NIR imaging was carried out on Maestro *in vivo* imaging system. NIR intensity was measured and calculated.

13. Detection of *in vivo* tumor therapeutic effect of $SELS^{DOX}$.

To monitor the tumor inhibition efficiency of $SELS^{DOX}$ and LS^{DOX}, we carried out *in vivo* tumor suppression studies. When the tumors had been allowed to develop to approximately 50-150 mm^3, the mice were randomly divided into four groups. The mice were injected intravenously with $SELS^{DOX}$, LS^{DOX} and DOX (200 μL) at a dose corresponding to 5 mg/kg of DOX (n=3). PBS was served as control. Administration was carried out on once every two days for seven times. And then the mice were observed for another five days. The tumor sizes and weights were recorded daily at the same time. Tumor sizes were measured by a vernier caliper. Tumor volume was calculated by the formula $(L \times W^2)/2$. L is for the longest and W is the shortest in tumor diameters (mm).

14. Slicing experiment and detection of the organs.

The mice were euthanized according to the animal protocol, and their tumors were immediately collected. To evaluate apoptosis levels in tumor cells, tumor tissue sections were stained by terminal deoxynucleotidyl transferased dUTP nick end labeling (TUNEL) assay. The nucleus were counterstained with Hoechst 33342 (nucleus indicator, 10 μg/mL, 200 μL) and examined by confocal microscope. Furthermore, to evaluate the necrosis levels, the heart, liver, spleen, lung, kidney and tumors were used for histopathology analysis (H&E) by light microscope.

15. Routine blood examination and blood chemistry determination after $SELS^{DOX}$ injection.

Six female BALB/c nude mice (6 weeks) purchased from *Vital River Laboratory Animal Center (Beijing, China)* were assigned to 2 groups (3 mice for each group): control group and $SELS^{DOX}$ group. The mice of $SELS^{DOX}$ group were treated with

$SELS^{DOX}$ NPs (200 μL) at a dose corresponding to 5 mg/kg of DOX by intravenous injection. The mice of control group were treated with 200 μL PBS. After 48 hours, the mice were sacrificed. The blood samples were collected by removing the eyeball of mice. Approximately 100 μL of blood was collected into anticoagulant tubes [potassium EDTA (Ethylenediaminetetraacetic acid)] for hematology examinations. The rest of the blood was placed at 4 °C for 4 h and subsequently centrifuged at 1500 rpm for 10 min to obtain blood plasma for blood biochemistry examinations.

16. Figure S1-S10.

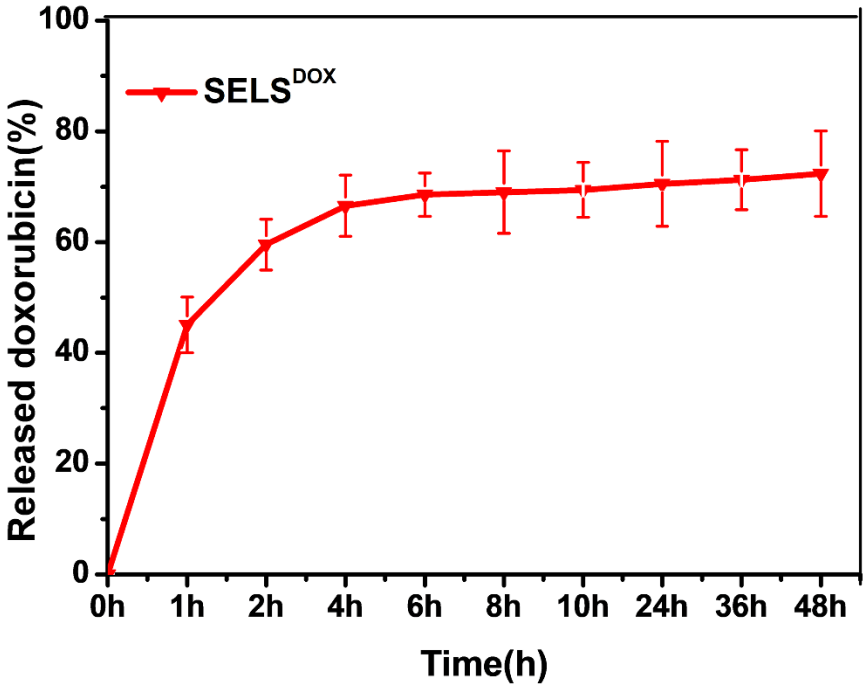

Figure S1. Drug release profile of $SELS^{DOX}$ in the dialysis process.

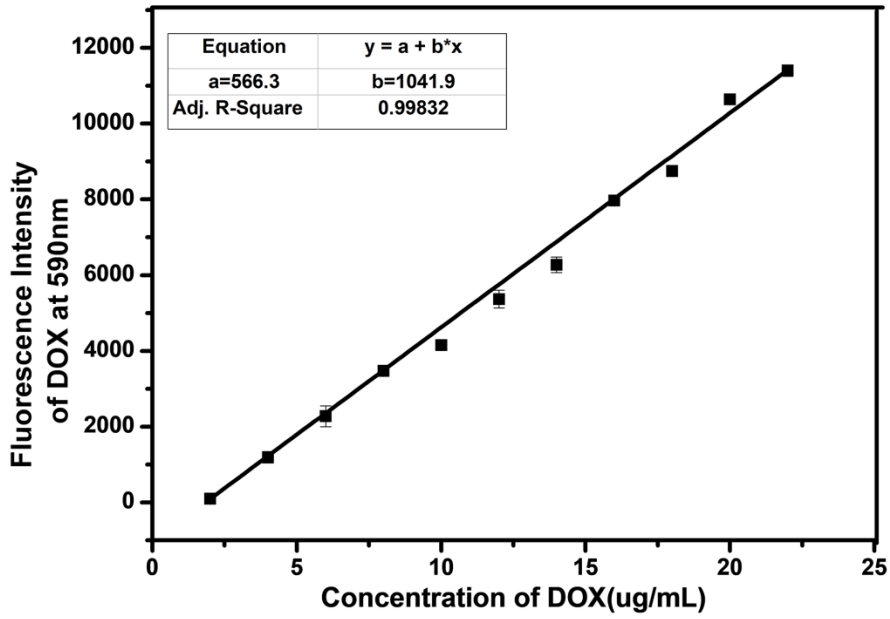

Figure S2. The standard curve of DOX intensity at different concentrations.

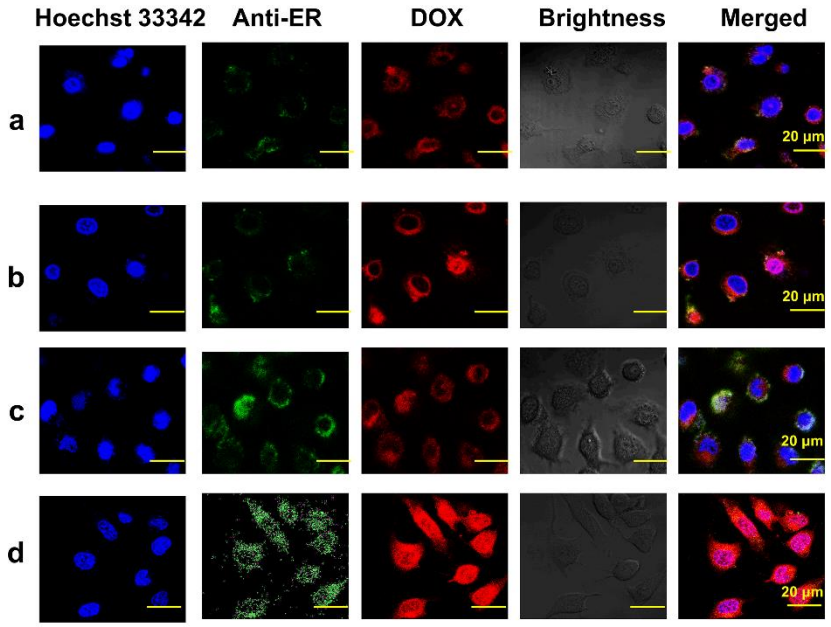

Figure S3. Confocal image of MCF-7 cells incubated with $SELS^{DOX}$ with different ratio of SPC, CHO, SP-DSPE-PEG, anti-ER-DSPE-PEG and DOX. The ratio of the five component is (a) 16:4:4:1:2, (b) 8:2:2:1:1, (c) 16:4:4:3:2 and (d) 8:2:2:2:1. When the ratio is 8:2:2:2:1, $SELS^{DOX}$ shows the best drug delivery efficiency.

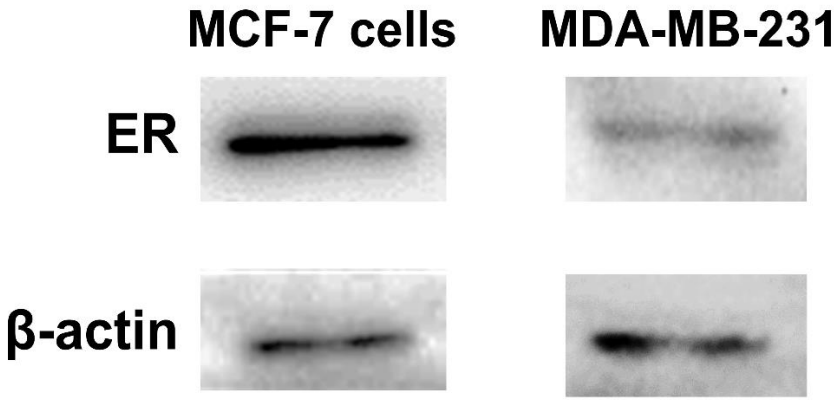

Figure S4. Western blot assay of the MCF-7 (ER over-expression) and MDA-MB-231 (ER low-expression) cell lysates. β-actin was used as a loading control.

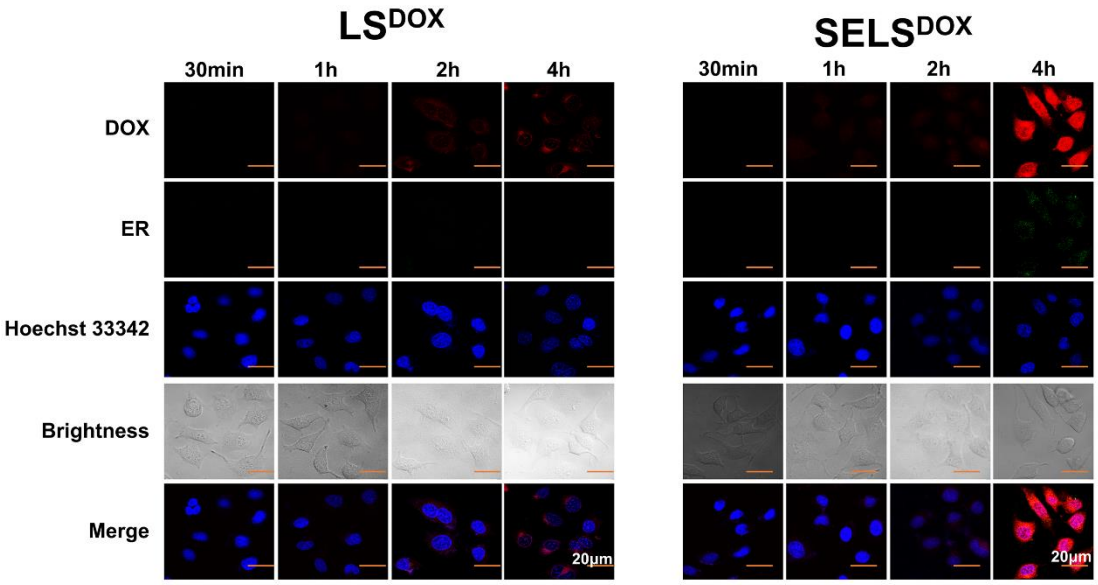

Figure S5. CLSM images of MCF-7 cells incubated with synergetic targeted nanoparticles ($SELS^{DOX}$) and untargeted nanoparticles (LS^{DOX}), containing equivalent DOX concentration of 5 μg/mL at the incubation time of 30 min, 1 h, 2 h and 4 h, respectively.

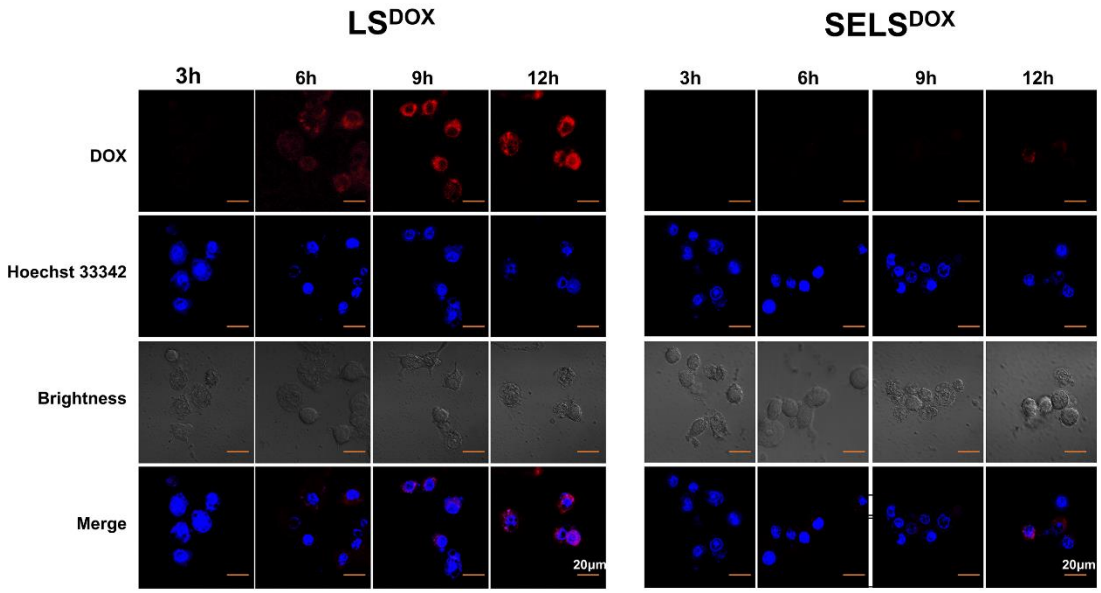

Figure S6. The phagocytosis effect of $SELS^{DOX}$ and LS^{DOX} by macrophage Raw 264.7 at the time point if 3 h, 6 h, 9 h and 12 h which were monitored by CLSM.

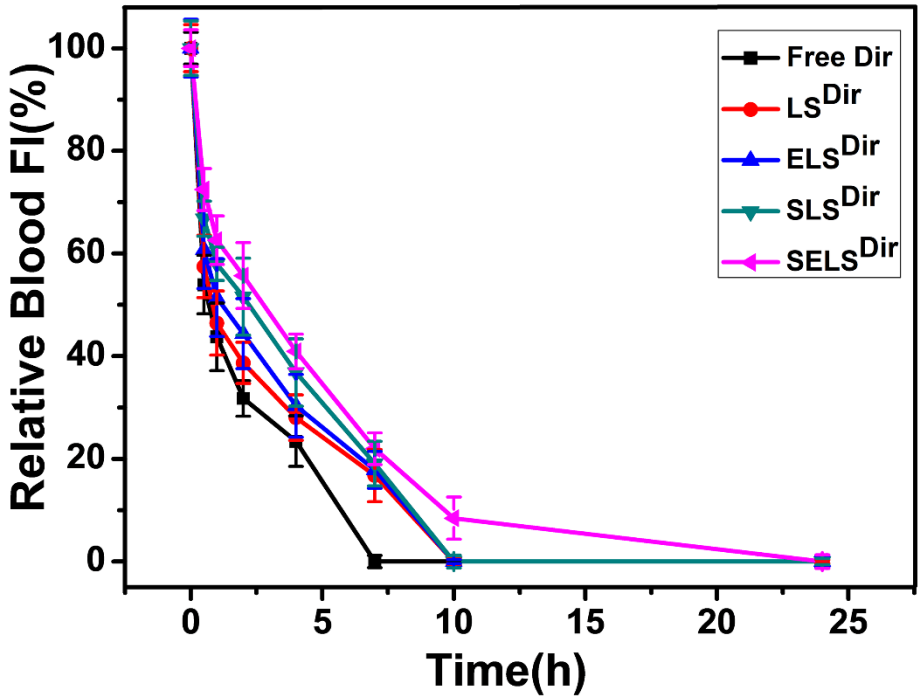

Figure S7. Blood circulation kinetics study of SELS. BALB/c nude mice were intravenously injected with $SELS^{Dir}$ as well as free Dir, LS^{Dir}, ELS^{Dir} and SLS^{Dir}, respectively (n = 3).

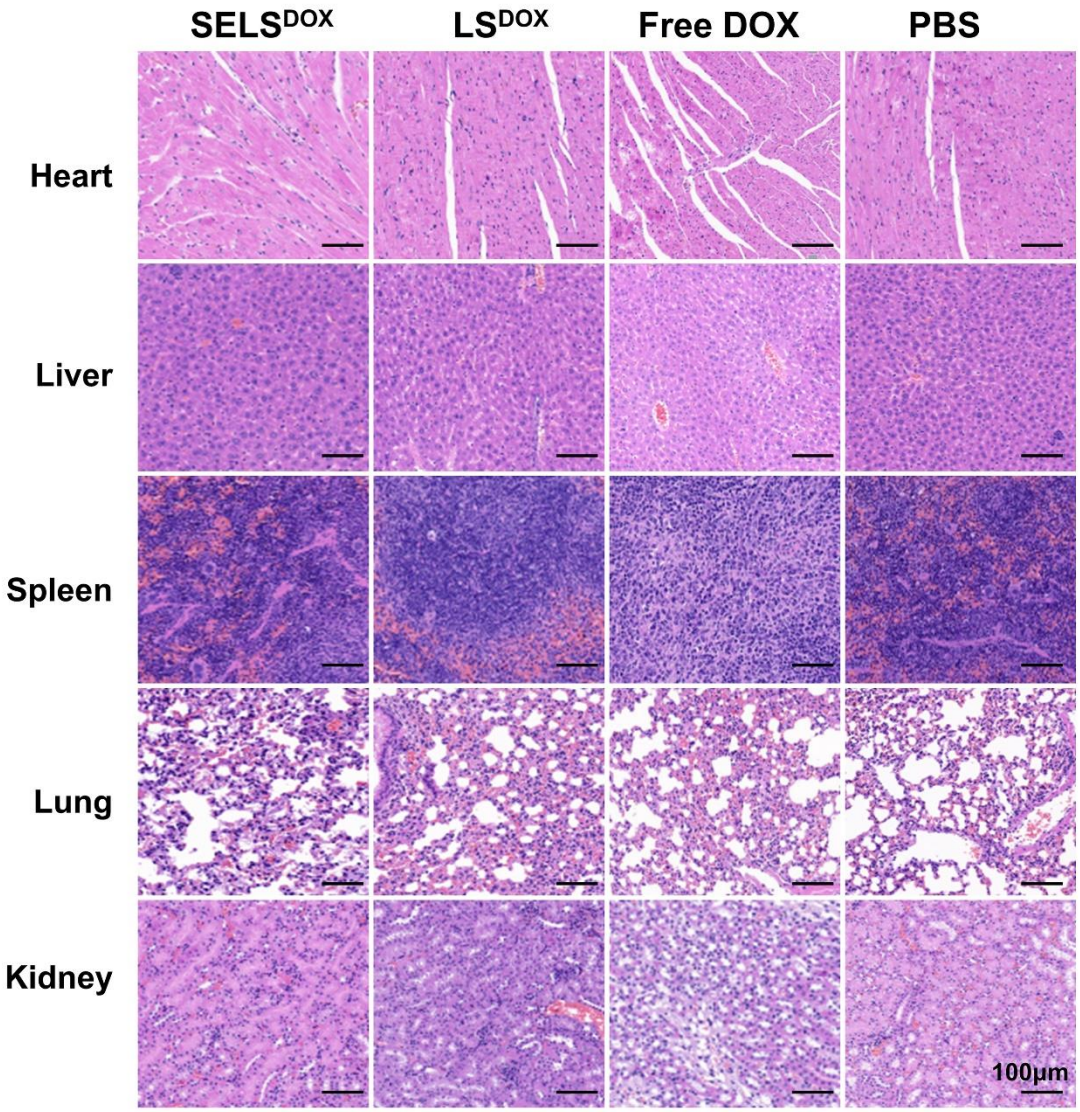

Figure S8. H&E staining analysis of paraffin section about heart, liver, spleen, lung, kidney of mice bearing MCF-7 xenograft after treatment with $SELS^{DOX}$, LS^{DOX}, and PBS.

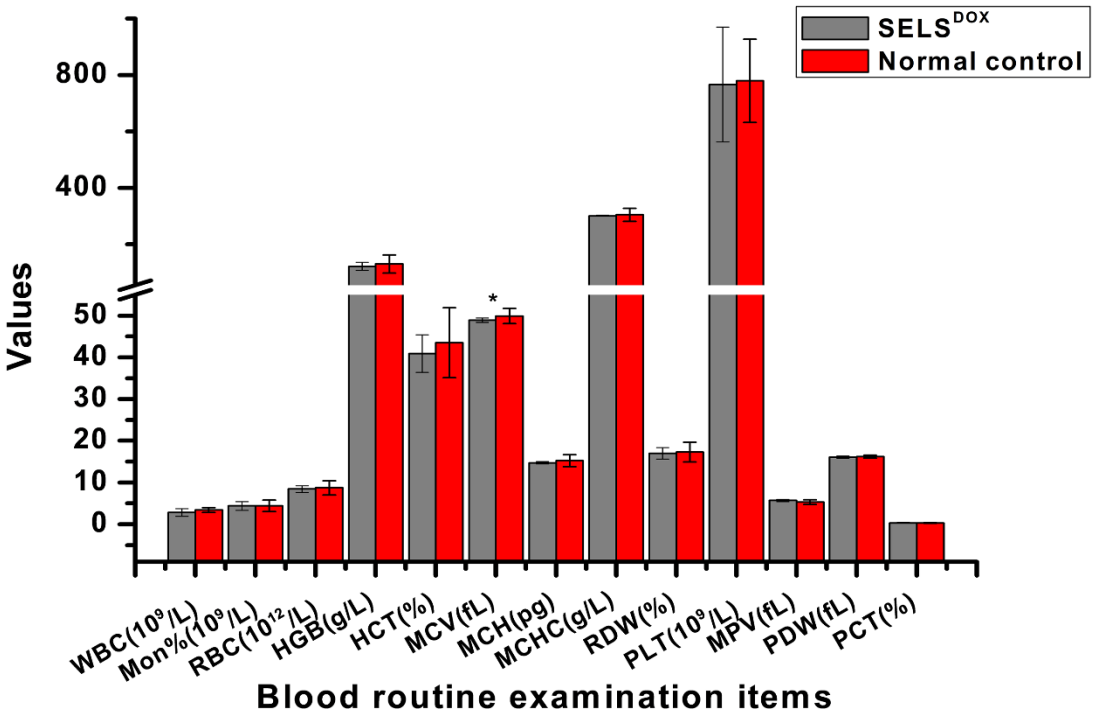

Figure S9. Blood routine examination analysis of mice after treatment with or without $SELS^{DOX}$ (n = 3 mice/group) (*P < 0.05, **P < 0.01, ***P < 0.001.).

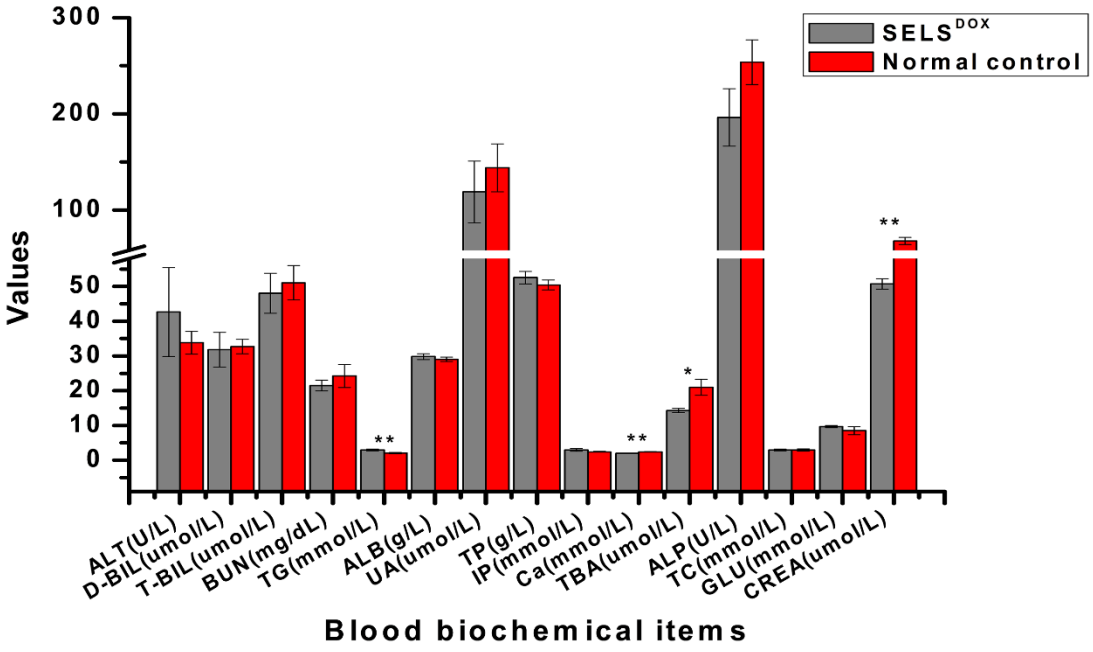

Figure S10. Blood biochemistry analysis of mice after treatment with or without $SELS^{DOX}$ (n = 3 mice/group) (*P < 0.05, **P < 0.01, ***P < 0.001.).