Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supporting Information

Peptide Functionalized Targeting Liposomes: for Nanoscale Drug Delivery towards Angiogenesis

Qiuju Han,[ab] Xiangqian Jia,[ab] Yixia Qian,[bc] Zihua Wang,[b] Shu Yang,[b] Yunhong Jia,*[a] Weizhi Wang,*[b] Zhiyuan Hu*[b]

[a.] Jinzhou Medical University, Jinzhou 121001, Liaoning, China

[b.] CAS Center for Excellence in Nanoscience, National Center for Nanoscience and Technology, Beijing 100190, P. R. China

[c.] University of Chinese Academy of Sciences, Beijing 100049, P. R. China

*Corresponding Authors' E-mail Addresses:
Yunhong Jia, jiayunhong2012@163.com
Dr. Weizhi Wang, wangwz@nanoctr.cn
Prof. Zhiyuan Hu, huzy@nanoctr.cn

Contents

1. Materials

Tentagel Resin was purchased from Rapp Polymere (Germany, loading 0.35 mmol/g). 9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, Wang resin, and 2-(1*H*-benzotriazole-1-*yl*)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU) were purchased from GL Biochem (China). Trifluoroacetic acid (TFA), thiazolyl blue tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), fluorescein isothiocyanate (FITC), Hoechst 33342, 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC) and streptavidin coated magnetic beads (1 μm) were from Sigma-Aldrich (USA). N-methyl morpholine (NMM), piperidine and N, N'-dimethylformamide (DMF) were all from Beijing Chemical Plant (China). 1,2-Ethanedithiol (EDT) was from Alfa Aesar (USA). Triisopropylsilane (Tips) was from Acros Organics (USA). Cyanogen bromide (CNBr) was from J&K Chemical (China). VEGFR2 protein was from Sino Biological Inc (Beijing, China). Biotin labeling kit was from SoluLink. Other reagents were all of analytical grade and used without further purification.

1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly (ethylene glycol)$_{2000}$]-maleimide (DSPE-PEG$_{2000}$-MAL) was purchased from Nanosoft Biotechnology LLC (USA). Soybean phosphatidylcholine (SPC, AR) and cholesterol (CHOL, AR) were purchased from A.V.T. Pharmaceutical Co., Ltd.. Doxorubicin hydrochloride (DOX) was supplied by Hisun Pharmaceutical Co. Ltd (Zhejiang, China).

Human umbilical vein endothelia cell line HUVEC, human embryonic kidney cell line 293T and human colonic adenocarcinoma cell line HT-29 were purchased from Cell Resource Center, Chinese Academy of Medical Sciences (China). Cell culture medium and fetal bovine serum were from WisentInc (Multicell, WisentInc, St. Bruno, Quebec, Canada) Culture dishes and plates were from Corning (Corning, New York, USA). HT-29 cells were maintained in RPMI 1640 medium with 10% fetal bovine serum and 1% penicillin. HUVEC and 293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and 1% penicillin. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 ºC.

2. Construction and synthesis of the OBOC peptide library towards VEGFR2

The details about synthessis process of the OBOC peptide library was shown in Fig. S1a and b. Fmoc strategy SPPS (solid phase peptide synthesis) was employed for synthesis of the OBOC library. Tentagel Resin (loading 0.35 mmol/g) was used as the solid phase support. The general sequence of the library is X_1 X_2 X_3 X_4 X_5 X_6 X_7 X_8 X_9 X_{10} X_{11} X_{12} X_{13} X_{14} X_{15} G M. X_1 represents any of Ser, Glu, Leu or Thr residue at N-terminal. X_2 is Ile, Lys, Asp, Pro or Ser, X3 is Asp, X_4 is Asn, His, Glu, Leu or Tyr, X_5 is Glu, Arg, Asp, Pro or Thr, X_6 is Trp or Leu, X_7 is Arg, Lys or His, X_8 is Lys, Arg, Glu, Leu or Ser, X_9 is Thr or Asn, X_{10} is Thr or Asn, X_{11} is Thr, Phe or Ser, X_{12} is Pro or Tyr, X_{13} is Leu, X_{14} is Ser, X_{15} is Pro or Phe. In the library, the sequence on each bead was randomly distributed so that the complexity of the peptide library was 1.08×10^6 and the redundancy of the library was five. As a result, peptide screening was carried out from 5.4×10^6 candidate beads. During the OBOC library synthesis, solid support beads were split equally in each cycle and different amino acids were added, separately. All the synthesis process of peptides were carried out in dehydrous DMF. In the deprotection step, 20% (v/v) piperidine in DMF was used to remove the Fmoc group and the deprotection time was 10 min. During the coupling step, the HBTU (4 mM) and Fmoc-amino acid (4 mM) were dissolved in DMF containing NMM (0.4 mM). The coupling time was 30 min. Qualitative Fmoc deprotection was confirmed by ninhydrin test (ninhydrin, phenol, VC 1:1:1 v/v). It means that amino acid coupling process was carried out in the "split" step while the deprotection process was carried out in "pool" step. All the above experiments were carried out in the solid phase peptide synthesis vessels with sieves in it. With magnetic conjugation assay, OBOC peptide beads could be trapped by VEGFR2 protein-biotin-

streptavidin labelling magnetic beads-assist screening at the magnetic field. An integrated lab-on-chip system performed the whole peptide screening process: positive peptide isolation, single bead trapping, and *in situ* sequencing. Based on one-bead-one-well microarray which is compatible with *in situ* matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS), sequencing was realized. For the *in situ* chemical cleavage in the microwells overnight, 30 mg/mL cyanogen bromide (BrCN) solution was used.

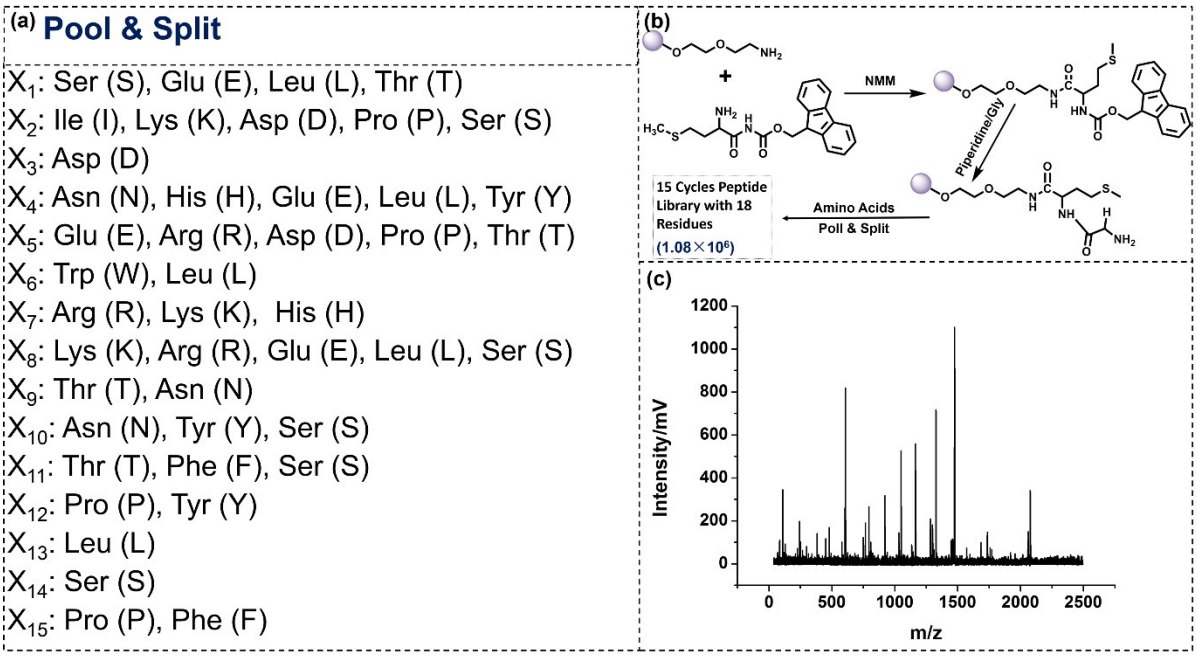

Fig. S1 (a) Amino acids at position of $X_{1\text{-}15}$ for pool & split method. (b) Synthesis process of the OBOC peptide library toward VEGFR2. (c) Representative MALDI-TOF-MS spectrum for the affinity peptide S1 cleaved off from positive beads.

3. Synthesis of peptides

When the positive sequence was determined by MALDI-TOF-MS (Bruker Daltonics; Fig. S1c), positive peptide (S1) was de novo synthesized. peptides were synthesized by solid-phase methods using a standard Fmoc-Chemistry. Wang Resin (Rapp Polymere, Germany, loading 0.34 mmol/g) was used as the solid phase support. All the synthesis process of peptides was carried out in dehydrous DMF. In the deprotection step, 20% (v/v) piperidine in DMF was used to remove the Fmoc group and the deprotection time was 10 min. During the coupling step, the HBTU (4 mM) and Fmoc-amino acid (4 mM) were dissolved in DMF containing NMM (0.4 mM). The coupling time was 30 min. Qualitative Fmoc deprotection was confirmed by a ninhydrin test (ninhydrin, phenol, VC 1:1:1 v/v). After elongation, cleavage reagents (92.5% TFA: 2.5% water: 2.5% EDT :2.5% Tips, v/v) were introduced into the vessel to cleave the side chain protecting group of each residue for 30 min in ice bath and then at room temperature for another 3 h. After peptides separated from the Wang resin, the mixture above were evaporated by vacuum rotary to remove the TFA. The crude peptides were then precipitated in cold anhydrous diethyl ether, collected by centrifuge and dried under vacuum. As is shown in Fig. S2a, b and c, the peptides were purified by preparative reversed-phase high performance liquid chromatography (HPLC) with a preparative reversed-phase Inertsil C18 HPLC column (ODS-3, 5 μm, 20 × 250 mm). A linear gradient of acetonitrile/water with 0.1% TFA respectively from 5/95 (v/v) to 70/30 (v/v) during 20 min，then 70/30 (v/v) to 90/10 (v/v) in 2 min and in this flow continue 1 min ,next return to 5%/95% (v/v) tilled for 2 min was used as the mobile phase. The separation was performed

with a flow rate of 1 mL/min and the monitoring wavelength was 220 nm using a UV detector (Waters 2535Q). As is shown in Fig. S2d, e and f,the purified peptides were determined by MALDI-TOF-MS using Microflex LRF system (Bruker Daltonics, Germany). MALDI-TOF-MS analysis was performed on a Bruker ULTRAFLEXTREME mass spectrometer equipped with a nitrogen laser (wavelength 337 nm, laser pulse uration 3 ns) with laser pulse energy between 0 and 100 μJ per pulse. The mass spectra were typically recorded at accelerating voltage of 19 kV, reflection voltage of 20 kV, and with laser pulse energy of 60 μJ. Each mass spectrum was acquired as an average of 500 laser shots.

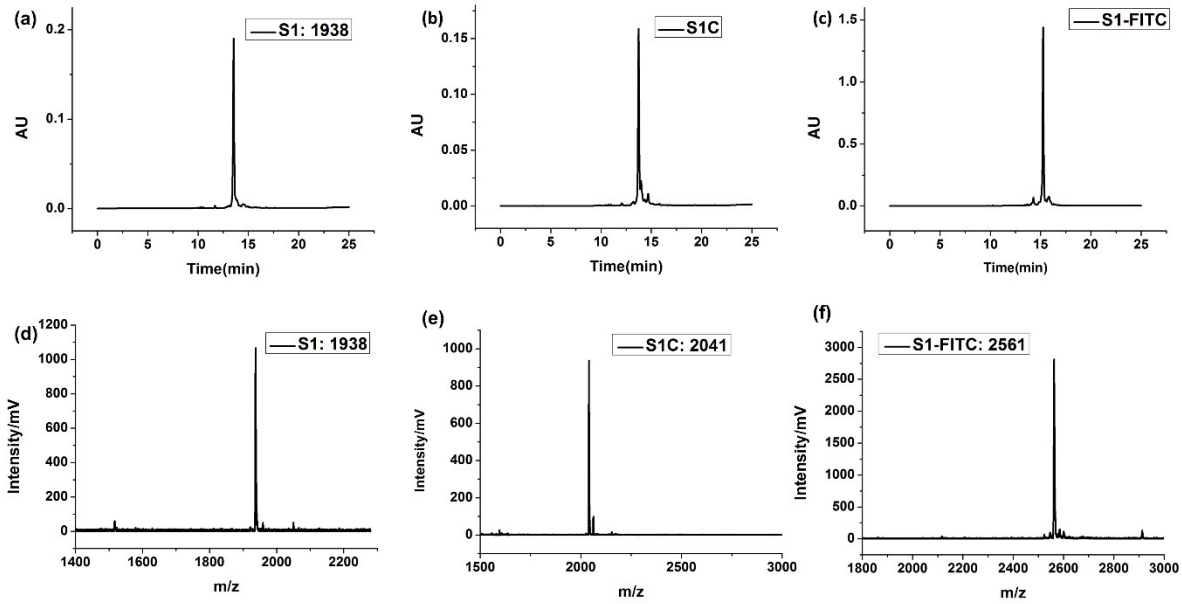

Fig. S2 (a-c) Purity identification for S1, S1C, S1-FITC by HPLC. (d-f) Mass spectrum of S1, S1C, S1-FITC detected by MALDI-TOF-MS.

4. SPRi for detection of the affinity peptide towards VEGFR2 protein

SPRi analysis was performed on a Plexera PlexArray HT system (Plexera LLC, Bothell, WA) using a bare gold SPRi chip (Nanocapture gold chips, with a gold layer of 47.5 nm thickness). The purified peptide S1C was printed onto the gold chip surface by the thiol group of the cystein residue. The printed chip was then incubated in 4 °C overnight in a humid box. The SPRi chip was washed and blocked using 5% (m/v) nonfat milk in PBS overnight before use. The SPRi analysis procedure follows the following cycle of injections: running buffer (PBST, baseline stabilization); sample (five concentrations of the protein, binding); running buffer (PBST, washing); and 0.5% (v/v) H_3PO_4 in deionized water (regeneration). VEGFR2 protein was diluted with PBST to concentrations of 7 nmol/L, 14 nmol/L, 28 nmol/L, 56 nmol/L and 128 nmol/L. Real-time binding signals were recorded and analyzed by PlexArray HT software.

5. Peptide ligands as probes for cancer cells imaging *in vitro*.

For HUVEC and 293T cells, approximately 1×10^5 mL^{-1} cells were seeded into culture dishes and cultured overnight. FITC-labeled peptide (S1-FITC) was dissolved in cold PBS at a concentration of 5.0×10^{-5} M. The cells were incubated with FITC-labeled peptide solution (200 μL, with Hoechst 33342 (10 μg/mL,200 μL) in the dark for 30 min at 4 °C. Finally, the cells were washed for three times with cold PBS. Confocal fluorescence imaging was performed on an Olympus FV1000-IX81 confocal laser scanning microscope (CLSM). A FV5-LAMAR 488 nm laser was the excitation source for FITC throughout the experiment, and

emission was collected between 520 and 620 nm. Hoechst 33342 was excited at 50 mW (ex: 405 nm, em: 472 nm). The objective lens used for imaging was a UPLSAPO 63× oil-immersion objective (Olympus).

6. Synthesis of S1-PEG$_{2000}$-DSPE

S1-PEG$_{2000}$-DSPE was synthesized by conjugation of S1C with DSPE-PEG$_{2000}$-MAL. Briefly, S1C and DSPE-PEG$_{2000}$-MAL (1:1, w/w) were dissolved in deionized water (total concentration: 12 mg/mL) , gently stirring at room temperature for 48 h continually. We used HPLC to monitor the reaction (Fig. S3). The peak of peptide was decreased along with the time. The reaction mixture were purified by dialysis (cut-off M.W. 3500 Da), and then lyophilized. The product was analyzed by MALDI-TOF-MS.

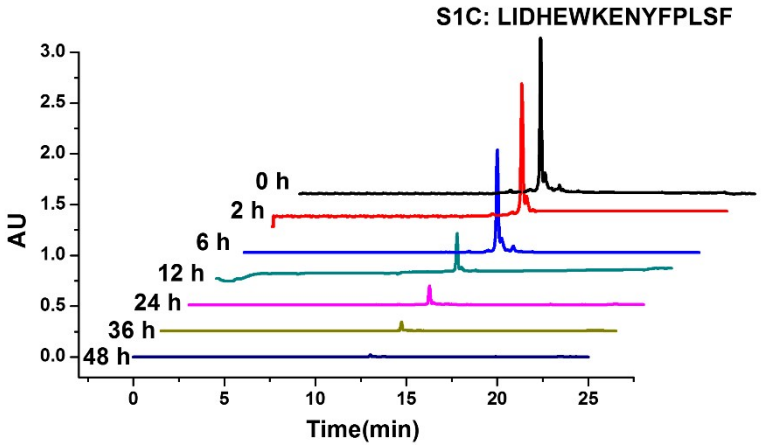

Fig. S3 Monitoring of the conjugation of S1C with DSPE-PEG$_{2000}$-MAL by HPLC.

7. Preparation and characterization of liposomes

Liposomes loading DOX were prepared by thin film dispersion method. S1-LS-DOX was prepared by mixing of soy phospholipids (SPC), cholesterol (CHO), S1-PEG$_{2000}$-MAL and DOX (SPC: CHO: S1-PEG$_{2000}$-DSPE: DOX 8: 2: 2: 1 w/w/w/w). Briefly, DOX (0.5 mg) was dissolved in 1 mL methanol at room temperature and mixed with S1-PEG$_{2000}$-DSPE (1 mg), cholesterol (1 mg), and soy phospholipids (4 mg) in 6 mL dichloromethane/methanol (v/v 2:1). The solvent was removed by vacuum rotary evaporation to form a dry drug-containing lipid film. The dried film was hydrated with phosphate buffer saline (PBS) at 60 °C for 15 min, then sonicated for 15 min by bath type sonicator. Finally, the liposome suspension was eluted by 0.22 μm sterile hyperfiltration membrane. With the same method, LS-DOX was prepared by SPC,CHO and DOX (SPC: CHO: DOX 4: 1: 1 w/w/w).

Particle size (hydration diameter) and zeta potential of liposomes were determined by dynamic light scattering (DLS) using a Zetasizer 5000 (Malvern Instruments, Malvern, Worcestershire, U.K.). The morphology of S1-LS-DOX and LS-DOX were determined using HITACHI Transmission Electron Microscope (HT7700 TEM, Japan) with 120 kV acceleration voltage. The process was described as following:

Put 8 μL liposome suspension onto carbon membrane support copper mesh for 1 min, and then moved away the residual liposome suspension. When the membrane was dried, 6 μL negative staining solution (1% uranyl acetate) was added to the membrane for 5 min. Following, remove the negative staining solution, and dry the membrane in the air. Finally, the samples were observed by TEM.

8. Encapsulation efficiency detection and i*n vitro* study of the liposomes

The standard curve of DOX concentration was measured with serial dilutions: 15, 7.5, 3.725, 1.8625, 0.9812 μg/mL (DOX dissolved in PBS). Fluorescence intensities of DOX solutions were measured at excitation wavelength of 480 nm and emission wavelength of 560-590 nm. Standard curve of DOX concentration with a linear regression coefficient (R^2) about 0.9977 was obtained (Fig. S4a). Next, the encapsulation efficiency (EE) of DOX in liposomes was also measured using following method. Briefly, unloaded DOX of liposome suspension 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 ($W_{\text{encapsulation}}$) and unloaded DOX (W_{unloaded}). Encapsulation efficiency (EE) was calculated according to the formula:

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

The encapsulation efficiency of S1-LS-DOX was about 83.49% and LS-DOX was about 81.26%.

The required quantity of DOX loaded liposomes were transferred into a dialysis bag (cut-off M.W. 3500). 1mL free DOX , LS-DOX and S1-LS-DOX were placed into the dialysis bags separately. Then the dialysis bags were introduced into 40 mL PBS buffer solution with 0.5% (v/v) Tween-80. And then the release systems were gently shaken at 37 °C. To estimate the amount of drug release, the fluorescence intensity of drug in the release medium at each sampling point was measured by infinite M200 microplate reader (Tecan, Durham, USA). Fig. S4b showed the cumulative release of free DOX from S1-LS-DOX and LS-DOX at pH 7.4. The release rate of DOX was high inshort time. However, the release rates of S1-LS-DOX and LS-DOX were silimar.

Besides, the stability test of liposomes *in vitro* was also carried out at 37 °C. S1-LS-DOX, LS-DOX and free DOX in PBS containing 10% fetal bovine serum were filtered by 0.22 μm filter membrane at 0 h, 24 h and 48 h successively. The fluorescent intensity of DOX was recorded by infinite M200 microplate reader. The relative fluorescence intensity of DOX released from S1-LS-DOX and LS-DOX showed little change (Fig. S4c).

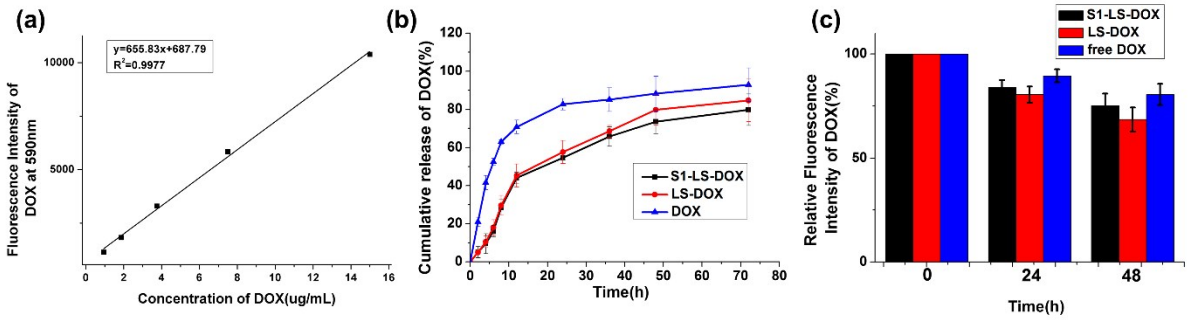

Fig. S4 (a) Standard fluorescence intensity curve of DOX in PBS. (b) *In vitro* release of DOX from S1-LS-DOX, LS-DOX and free DOX (n=3). (c) *In vitro* stability of S1-LS-DOX and LS-DOX were measured by relative fluorescence intensity of DOX (free DOX was control)

9. Confocal fluorescence imaging of cancer cells

For HUVEC and 293T cells, approximately 1×10^5 mL^{-1} cells were seeded into 35 mm microscope dishes and cultured overnight. Liposomes (S1-LS-DOX and LS-DOX) were dissolved in cell culture medium to 30 μg/mL. 293T cells were incubated with Hoechst 33342 (nucleus indicator, 10 μg/mL, 200 μL) at 37 °C for 15 min, then incubated with S1-LS-DOX (30 μg/mL, 200 μL) at 37 °C for 15 min. HUVEC cells were incubated with Hoechst 33342 (10 μg/mL, 200 μL) at 37 °C for 15 min, then incubated with different liposomes solution (30 μg/mL, 200 μL) at 37 °C for 15 min, separately. To investigate cell binding specificity

of S1-LS-DOX towards HUVEC, the competition experiment was carried out. After HUVEC were treated with excess of free S1 (1 mg/mL, 300 μL) for 15 min to block the binding sites, the cells were incubated with Hoechst 33342 (10 μg/mL, 200 μL) and S1-LS-DOX (30 μg/mL, 200 μL) for 15 min at 37 °C. 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).

Cellular uptake of S1-LS-DOX and LS-DOX at different time were investigated by CLSM qualitatively. HUVEC cells were seeded into 35 mm microscope dishes and cultured overnight. Cells were incubated with Hoechst 33342 (10 μg/mL, 200 μL) for 15 min first. Then cells were incubated with S1-LS-DOX (30 μg/mL, 200 μL) for 10 min, 15 min, 30 min, 60 min or 120 min at 37 °C. Cells were washed with PBS for three times and observed by confocal laser scanning microscope. With the same method, HUVEC cells incubated with LS-DOX (30 μg/mL, 200 μL) was carried out as control.

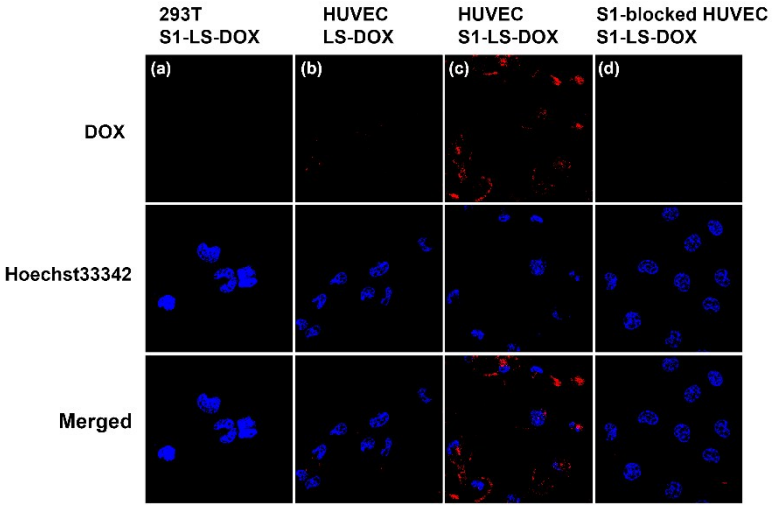

Fig. S5 CLSM images of cells incubated with S1-LS-DOX or LS-DOX for 15 min. (a) 293T cells incubated with S1-LS-DOX. (b) HUVEC cells incubated with LS-DOX. (c) HUVEC cells incubated with S1-LS-DOX. (d) S1-blocked HUVEC cells incubated with S1-LS-DOX.

The cellular uptake of DOX-loaded liposomes was studied by flow cytometry analysis. Typically, HUVEC cells were seeded into six-well plates at a density of 5×10^5 cells/well and cultured at 37 °C for 24 h, respectively. Prior to the experiment, cells were washed twice with PBS to remove the remnant growth medium, and then incubated in serum-free medium containing various DOX formulations at the final DOX concentration of 30 μg/mL. After 30 min incubation of S1-LS-DOX, LS-DOX or free DOX, the cells were washed for three times with cold PBS and then re-suspended in 500 μL of PBS. The DOX fluorescence intensity was measured by a flow cytometer (Becton Dickinson FACS Calibur, Mountain View, USA).

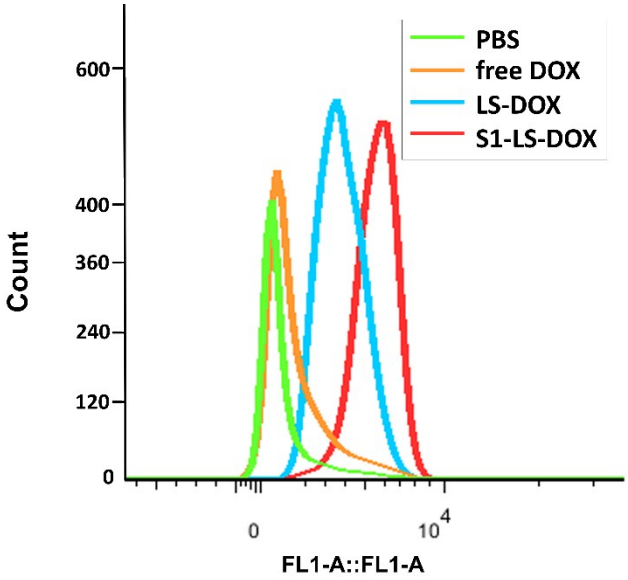

Fig. S6 Flow cytometric measurement of DOX fluorescence intensity in HUVEC cells incubated with PBS (as control), free DOX, LS-DOX and S1-LS-DOX.

10. Inhibition ratio of cells studies

In DOX concentration-dependent experiment (Fig. S7a), HUVEC cells were seeded at 5×10^3 cells per well in 96-well plates, and pre-incubated for 24 h, then incubated with S1-LS-DOX, LS-DOX or free DOX for 48 h at doxorubicin concentrations ranging from 0. 1 to 80 μg/mL (100 μL). In time-dependent experiment (Fig. S7b), HUVEC cells were seeded at a density of 5×10^3 cells per well in flat bottom 96-well plates and incubated for 24 h. Thereafter, S1-LS-DOX, LS-DOX or free DOX (80 μg/mL, 100 μL) were added in the wells of the plates and then exposed for 2, 4, 6, 8, 10 or 12 h. After that, fresh basic medium (100 μL) took the places of S1-LS-DOX, LS-DOX or free DOX for 24 h incubation in incubator containing 5% CO_2 at 37 °C. Then medium was replaced with 100 μL 0.5 mg/mL MTT and after 3 h the MTT solution was replaced with 150 μL DMSO solution. Untreated cells in medium were used as control. The absorbance was measured at 570 nm with a reference wavelength of 630 nm using an Infinite M200 microplate reader (Tecan, Durham, USA). All experiments were carried out with four replicates. The bar charts were shown in Fig. S7.

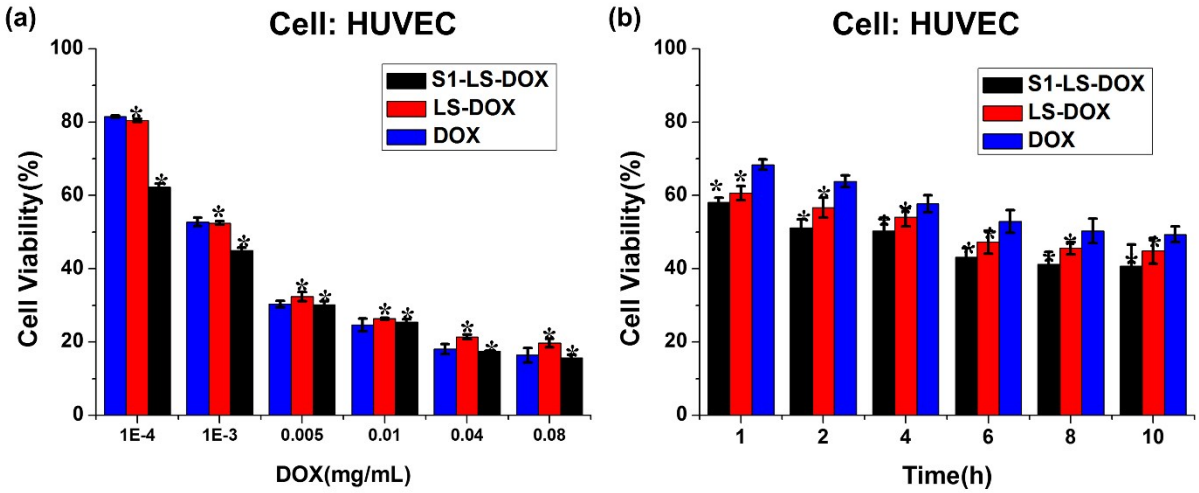

Fig. S7 *In vitro* cytotoxicity of free DOX, LS-DOX and S1-LS-DOX. (a) MTT assay for HUVEC cells incubated with different DOX formulations at different concentrations. (b) MTT assay for HUVEC cells

incubated with different DOX formulations for different times.

11. *In vivo* biodistribution of S1-LS

Female BALB/c nude mice about 18 g were purchased from Vital River Laboratory Animal Center (Beijing, China), and kept under specific pathogen-free 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 (S.C.) injection of 1×10^7/mL HT-29 cells (100μL) to the right hind leg of the approximate 6 week-old female BALB/c nude mice. 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbonyaineiodide (DiR) with emission maxima centered at 748 nm were purchased from Invitrogen. Tumor growth was measured periodically and until diameters of the tumors reached to grow to about 7 mm (14 weeks old on arrival). Mice were injected via the caudal vein with, LS-DiR and S1-LS-DiR (200 μL) at dose corresponding to 1 μg/mL DiR. PBS was served as control. Near-infrared imaging was carried out after 8 h administration by Maestro *in vivo* spectrum imaging system (Cambridge Research & Instrumentation, Woburn, MA). Then, organs were excised for *ex vivo* fluorescence imaging. All images were taken with ten-second exposure time to ensure consistency in the data.