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Supplementary Information

Acid/Redox Dual-Activated Liposomes for Tumor-Targeted Drug Delivery and Enhanced Therapeutic Efficacy

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Materials.

L-Glutamic acid, stearyl alcohol, *p*-toluene sulfonic acid (TsOH), triethylamine (TEA) and N, N-dicyclohexylcarbodiimide (DCC) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). N-tert-butoxycarbonyl-N-(imidazole)-(4-toluenesulfonyl)-L-histidine (Boc-L-His(Tos)-OH) was obtained from GL Biochem Co., Ltd. (Shanghai, China). 1-Hydroxybenzotrizole (HOBt), hexahydrophthalic anhydride (HHPA), N-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) and succinic anhydride (Suc) were purchased from Aladdin Reagent Inc. (Shanghai, China). DiR (1,1'-dioctadecyl-3,3,3',3'-tetramethyl indotricarbocyanine iodide), amiloride, sucrose and nystatin were bought from Sigma (St. Louis, MO, USA). Doxorubicin hydrochloride (DOX) was offered from Beijing HuaFeng United Technology Co., Ltd. (Beijing, China). Soy phosphatidylcholine (SPC) was purchased by Taiwei Pharmaceutical Co., Ltd. (Shanghai, China). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was purchased from Avanti Polar Lipids Inc. (USA). RPMI 1640 medium (1640, Hyclone®), LysoTracker Green (Life Technologies®) and trypsin (Hyclone®) were purchased from Pufei Bio-Technology Co., Ltd. (Shanghai, China). Penicillin-streptomycin solution (Hyclone®), fetal bovine serum (FBS), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) and phosphate buffered saline (PBS, Hyclone®) were bought from Sunshine Biotechnology Co. Ltd. (Nanjing, China). All other reagents were analytical grade.

Synthesis and Characterization of HH-SS-E2C$_{14}$.

2-[2-(2-carboxylcyclohexylformamido)-3,12-dioxy-1-(1H-imidazolyl-4)-7,8-dithio-4,11-diazapenta decylamide]-glutaric acid ditetradecanol-diester (designated as **HH-SS-E2C$_{14}$**) was synthesized as

presented in Scheme S1.

L-Glutamic acid (2.9 g, 19.7 mmol) and TsOH (2.22 g, 11.7 mmol) were dissolved in methylbenzene (50 mL), and refluxed for 1 h at 110 ^{o}C. Tetradecyl alcohol (5.0 g, 23.3 mmol) was added to the solution, followed by stirring for 12 h under reflux. The reaction mixture was evaporated with vacuum distillation to remove methylbenzene and then dissolved in dichloromethane (DCM). The DCM solution was successively washed with the 5% (w:v) $NaHCO_3$ solution (10 mL × 2) and the saturated NaCl solution (10 mL × 1), and then evaporated. 1,5-tetradecanol-glutamic acid (**E2C$_{14}$**) was recrystallized from methanol (CH_3OH) (10 mL) to obtain a white powder with a yield of 53.4%.

Cystamine dihydrochlorate (3.0 g, 13.3 mmol) was dissolved in CH_3OH (150 mL). TEA (5.79 mL, 39.9 mmol) and $(Boc)_2O$ (2.9 g, 13.3 mmol) were successively added to the solution, followed by stirring for 5 h at room temperature. After the reaction solution was evaporated, the obtained powder was dissolved in NaH_2PO_4 (60 mL, 1 M) and extracted by ethyl ether (60 mL × 2). The water layer was adjusted to pH 9 and extracted by ethyl acetate (40 mL × 2). The organic layers were mixed, washed with the distilled water (60 mL × 2), and then evaporated. 1-(tertiary butyloxycarbonyl) cystamine (AED(Boc)) was obtained with a yield of 46.8% by drying with anhydrous Na_2SO_4.

AED(Boc) (1.57 g, 6.23 mmol) was dissolved in DCM (80 mL). Suc (0.62 g, 6.23 mmol) and dimethylamino pyridine (0.23 g, 1.87 mmol) were then dissolved in DCM (15 mL) and added into the AED(Boc) solution, followed by stirring at room temperature for 5 h. The reaction mixture was washed with $KHSO_4$ (50 mL × 2) and the organic layer with the saturated NaCl solution (60 mL × 1), and then evaporated. 1-(tertiary butyloxycarbonyl)-8-(3-carboxylpropionyl)cystamine (AED(Boc)-Suc) was obtained with a yield of 98.2% by drying with anhydrous Na_2SO_4.

AED(Boc)-Suc (2.12 g, 6.0 mmol), EDC·HCl (2.31 g, 12.0 mmol) and HOBt (1.62 g, 12.0 mmol) were dissolved in DCM (80 mL) with stirring at room temperature for 3 h. $E2C_{14}$ (3.24 g, 6.0 mmol) and TEA (3.36 mL, 24.0 mmol) were dissolved in $CHCl_3$ (40 mL). The above two solutions were mixed and stirred for 12 h. The reaction mixture was successively washed with the distilled water (80 mL × 2) and the saturated saline solution (60 mL × 1), and then evaporated. 2-(2,2-dimethyl-4,13-diketo-3-oxa-8,9-dithio-5,12-diazadithioamide) glutaric acid ditetradecanoldiester (**AED(Boc)-Suc-2EC$_{14}$**), a white powder with a yield of 77.9%, was obtained by column chromatography separation with the eluent of DCM and CH_3OH (60:1, v:v), followed by the rotary evaporation.

AED(Boc)-Suc-$2EC_{14}$ (4.1 g, 4.7 mmol) was dissolved in ethyl acetate solution (200 mL) saturated with HCl, followed by stirring at 0 ^oC for 12 h. 2-(9-oxa-4,5-dithio-1,8-diazadialkyl amide) glutaric acid ditetradecanoldiester hydrochlorate (**AED-Suc-2EC$_{14}$**), a white wax with a yield of 86.8%, was obtained by filtration.

Boc-L-His(Tos)-OH (0.81 g, 2.0 mmol), EDC·HCl (0.76 g, 4.0 mmol) and NHS (0.45 g, 4.0 mmol) was dissolved in $CHCl_3$ (40 mL) with stirring at room temperature for 3 h. AED-Suc-$2EC_{14}$ (1.6 g, 2.0 mmol) and TEA (1.1 mL, 8.7 mmol) was also dissolved in $CHCl_3$ (20 mL). The above two solutions were mixed and stirred at room temperature for 12 h, and then the mixture was washed by the distilled water (50 mL × 2) and the saturated saline solution. 2-(2,2-dimethyl-4,7,16-triketo-6-((1-para-tosyl-imidazolyl-4-)methyl)-3-oxa-11,12-dithio-5,8,15-triazanonadecyl) glutaric acid ditetradecanoldiester (**His(Boc)(Tos)-AED-Suc-E2C$_{14}$**), a white powder with a yield of 74.1%, was obtained by column chromatography separation with the eluent of DCM and CH_3OH (50:1, v:v), followed by the rotary evaporation.

His(Boc)(Tos)-AED-Suc-$E2C_{14}$ (1.51 g, 1.3 mmol) was dissolved in THF (40 mL) and HOBt

(2.10 g, 15.6 mmol) was added into this solution and stirred at 40 ^{o}C for 5 h. The reaction solvents were removed by rotary evaporation and the white solid was obtained. 2-(2,2-dimethyl-4,7,16-triketo-6-((1-imidazolyl-4)methyl)-3-oxa-11,12-dithio-5,8,15-triazanonadecyl) glutaric acid ditetradecanoldiester (**His(Boc)-AED-Suc-E2C$_{14}$**), a amber wax solid with a yield of 95.4%, was obtained by column chromatography separation with the eluent of DCM and CH_3OH (15:1, v:v), followed by the rotary evaporation.

His(Boc)-AED-Suc-E2C$_{14}$ (1.25 g, 1.2 mmol) was dissolved in the HCl-saturated ethyl acetate (25 mL) with stirring at 0 ^{o}C for 12 h. 2-(3,12-diketo-2-((1H-imidazolyl-4)methyl)-7,8-dithio-1,4,11-triazapentadecyl) glutaric acid ditetradecanoldiester (**H-SS-E2C$_{14}$**),[1] a white wax solid with a yield of 78.7%, was obtained by filtration.

His(Boc)(Tos)-AED-Suc-E2C$_{14}$ (1.7 g, 1.5 mmol) was dissolved in the mixed solutions of trifluoroacetic acid (TFA) (3 mL) and DCM (3 mL) with stirring at room temperature for 4 h. The reaction mixture was processed by extraction with DCM (15 mL × 3) after the pH was adjusted to neutrality with the 5% $NaHCO_3$ solution. The obtained organic solvents were removed by rotary evaporation and 2-(3,12-dicarbonyl-2-((1-para-tosyl-imidazolyl-4)methyl)-7,8-dithio-1,4,11-triazapentadecyl) glutaric acid ditetradecanoldiester (**His(Tos)-AED-Suc-E2C$_{14}$**), a white powder with a yield of 71.0%, was obtained by column chromatography separation with the eluent of DCM and CH_3OH (25:1, v:v), followed by rotary evaporation.

His(Tos)-AED-Suc-E2C$_{14}$ (1.0 g, 0.94 mmol), HHPA (0.15 g, 0.94 mmol) and DMAP (0.04 g, 0.29 mmol) were dissolved in DCM (45 mL) with stirring for 5 h. The reaction mixture was washed by 1 M $KHSO_4$ (20 mL × 2) and the organic layer by the saturated NaCl solution (20 mL × 1). The obtained white oily liquid (1.3 g, 1.08 mmol) after evaporation and HOBt (1.76 g, 13.0 mmol) were dissolved in tetrahydrofuran (THF) (30 mL) with stirring for 5 h at 40 ^{o}C. After evaporation of THF

in the reaction solution, the obtained white solid was purified by column chromatography separation with the eluent of DCM and CH_3OH (8:1, v:v), followed by rotary evaporation. 2-[2-(2-carboxylcyclohexylformamido)-3,12-dioxy-1-(1H-imidazolyl-4)-7,8-dithio-4,11-diazapenta decylamide]-glutaric acid ditetradecanol-diester (**HH-SS-E2C$_{14}$**), a white powder with a yield of 72.0%, was obtained after removal of organic solvents.

AED(Boc)-Suc-E2C$_{14}$: ^{1}H NMR (500 MHz, $CDCl_3$) δ: 4.57 (1H, m), 4.12 (2H, t, J=6.75), 4.06 (2H, t, J=6.80), 3.56 (2H, m), 3.43 (2H, m), 2.80 (4H, m), 2.57 (4H, m), 2.35 (2H, m), 2.19 (1H, m), 1.99 (1H, m), 1.62 (4H, m), 1.44 (9H, s), 1.26(44H, m), 0.88 (6H, m). HRMS, ESI$^+$, m/z: Calcd for $C_{46}H_{87}N_3NaO_8S_2$ $(M+Na)^+$ 896.5827; found, 896.5845; IR (film, cm^{-1}): 3363, 3331, 2919, 2850, 1732, 1682, 1649, 1525, 1468, 1278, 1192, 1097, 966, 721, 632.

AED-Suc-E2C$_{14}$: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.55 (1H, m), 4.07 (4H, m), 3.56 (2H, m), 3.56 (2H, m), 2.79 (4H, m), 2.54 (4H, m), 2.39 (2H, m), 1.97 (2H, m), 1.84 (2H, m), 1.57 (4H, m), 1.24 (44H, m), 0.87 (6H, m). HRMS, ESI$^+$, m/z: Calcd for $C_{41}H_{80}N_3O_6S_2$ $(M+H)^+$ 774.5483; found, 774.5492; IR (film, cm^{-1}): 3308, 2961, 2918, 2850, 1739, 1639, 1537, 1469, 1420, 1262, 1199, 1095, 1021, 801, 719, 698.

His(Boc)(Tos)-AED-Suc-E2C$_{14}$: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.94 (1H, s), 7.35-7.83 (4H, m), 7.14 (1H, s), 4.58 (1H, m), 4.46 (1H, m), 4.08 (4H, m), 3.49 (4H, m), 3.00 (2H, m), 2.81 (2H, m), 2.65 (1H, m), 2.59 (4H, m), 2.44 (3H, s), 2.38 (2H, m), 2.18 (1H, m), 1.95 (2H, m), 1.60 (4H, m), 1.41 (9H, m), 1.26 (44H, m), 0.88 (6H, m). MS, ESI$^+$, m/z: Calcd for $C_{59}H_{100}N_6NaO_{11}S_3$ $(M+Na)^+$ 1187.7; found, 1187.7; IR (film, cm^{-1}): 3314, 3068, 2921, 2851, 1734, 1655, 1530, 1469, 1380, 1330, 1248, 1174, 1093, 815, 721, 676, 592, 541.

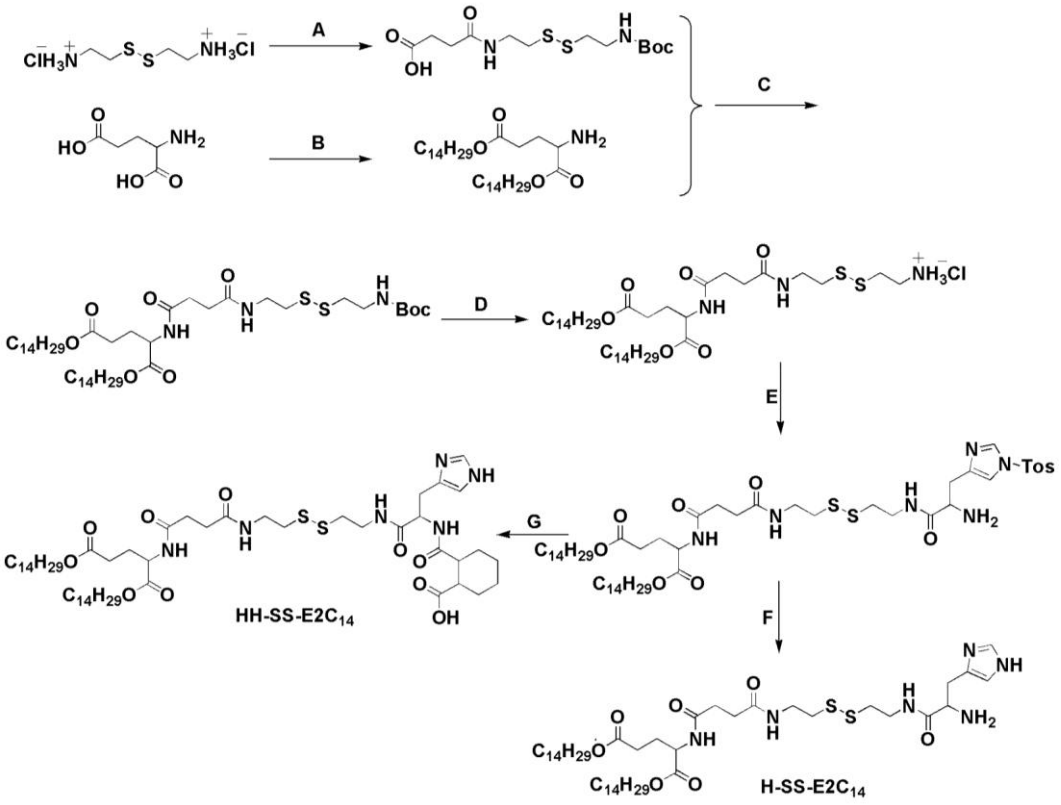
His(Boc)-AED-Suc-E2C$_{14}$: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.98 (1H, s), 7.64(1H, m), 4.46 (2H, m), 3.97 (4H, m), 3.42 (4H, m), 3.02 (2H, m), 2.57 (8H, m), 2.30 (2H, m), 1.87-2.12 (2H, m),

1.52 (4H, m), 1.28 (9H, m), 1.18 (44H, m), 0.80 (6H, m). HRMS, ESI^+, m/z: Calcd for $C_{52}H_{95}N_6O_9S_2$ $(M+H)^+$ 1011.6596; found, 1011.6602; IR (film, cm^{-1}): 3315, 2956, 2919, 2851, 1733, 1647, 1529, 1468, 1397, 1368, 1331, 1251, 1194, 1035, 816, 721, 684, 569.

H-SS-E2C$_{14}$: ^1H NMR (300 MHz, $CDCl_3$) δ: 7.59 (1H, s), 6.88 (1H, m), 4.56 (1H, m), 4.09 (4H, m), 3.54 (5H, m), 3.03 (2H, m), 2.79 (4H, m), 2.60 (4H, s), 2.40 (2H, m), 2.19 (1H, m), 2.00 (1H, m), 1.63 (4H, m), 1.27 (44H, m), 0.89 (6H, m). HRMS, ESI^+, m/z: Calcd for $C_{47}H_{86}N_6NaO_7S_2$ $(M+Na)^+$ 933.5892; found, 933.5910; IR (film, cm^{-1}): 3245, 2922, 2852, 1738, 1677, 1640, 1560, 1467, 1365, 1193, 1082, 1034, 819, 721, 624.

His(Tos)-AED-Suc-E2C$_{14}$: ^1H NMR (300 MHz, $CDCl_3$) δ: 7.89 (1H, s), 7.36-7.84 (4H, m), 7.14 (1H, s), 4.57 (1H, m), 4.08 (4H, m), 3.71 (1H, m), 3.53 (4H, m), 3.03 (1H, m), 2.83 (3H, m), 2.72 (2H, m), 2.58 (4H, s), 2.45 (3H, s), 2.38 (2H, m), 2.18 (4H, m), 1.97 (1H, m), 1.62 (4H, m), 1.26 (41H, m), 0.86 (6H, m). HRMS, ESI^+, m/z: Calcd for $C_{54}H_{93}N_6O_9S_3$ $(M+H)^+$ 1065.6161; found, 1065.6172; IR (film, cm^{-1}): 3312, 2955, 2920, 2851, 2361, 2342, 1743, 1639, 1536, 1468, 1376, 1327, 1273, 1191, 1170, 1096, 812, 721, 687, 589, 542.

HH-SS-E2C$_{14}$: ^1H NMR (300 MHz, $CDCl_3$) δ: 8.29 (1H, m), 7.63(1H, m), 5.02 (1H, m), 4.55 (1H, m), 4.08 (4H, m), 3.53 (4H, m), 3.37 (1H, m), 3.25 (1H, m), 2.99 (1H, m), 2.82 (4H, m), 2.62 (4H, s), 2.40 (2H, m), 2.18 (1H, m), 2.02 (3H, m), 1.98 (3H, m), 1.26-1.90 (56H, m), 0.89 (6H, m). HRMS, ESI^+, m/z: Calcd for $C_{55}H_{97}N_6O_{10}S_2$ $(M+H)^+$ 1065.6702, found, 1065.6713; IR (film, cm^{-1}): 3313, 2920, 2851, 1734, 1647, 1532, 1467, 1400, 1331, 1129, 1092, 1034, 817, 721, 628.



Scheme S1. Synthesis of H-SS-E2C_{14} and HH-SS-E2C_{14}. Reagents and reaction conditions: A: $(Boc)_2O$, Suc; B: TsOH, $C_{14}H_{29}OH$; C: EDC·HCl, HOBt; D: EtOAc; E: Boc-L-His(Tos)-OH, TFA; F: HOBt; G: HHPA, HOBt.

Synthesis and Characterization of HH-E2C_{14}.

2-[2-[(2-Carboxy-cyclohexanecarbonyl)-amino]-3-(1H-imidazol-4-yl)-propionylamino]-pentanedioic acid ditetradecyl ester (**HH-E2C_{14}**) was synthesized as presented in Scheme S2.

Boc-L-His(Tos)-OH (2.28 g, 5.6 mmol), EDC·HCl (1.71 g, 8.9 mmol) and NHS (1.02 g, 8.9 mmol) were dissolved in $CHCl_3$ (60 mL). E2C_{14} (3.0 g, 5.6 mmol) and TEA (2.3 mL, 16.7 mmol) were also dissolved in $CHCl_3$ (20 mL). The mixture of the two solutions above was stirred at room temperature for 12 h. Afterward, the reaction mixture was successively washed by the distilled water (50 mL × 2) and the saturated NaCl solution (50 mL × 1). After removal of the solvents, the obtained white solid was dissolved in the mixture of TFA (5 mL) and DCM (5 mL) with stirring at

room temperature for 4 h. Subsequently, the pH of the reaction mixture was adjusted to neutrality by the 5% $NaHCO_3$ and extracted by DCM (15 mL × 3). The obtained organic layers were mixed and then washed by the saturated NaCl solution. After evaporation of DCM, the obtained amber solid was purified by column chromatography separation with the eluent of DCM and CH_3OH (50:1, v:v), followed by rotary evaporation. 2-(2-amino-3-(1-tosyl-1-H-imidazolyl) triamino) glutaric acid ditetradecanol-diester (**His(Tos)-E2C$_{14}$**), a white solid with a yield of 63.7%, was obtained after removal of organic solvents.

His(Tos)-E2C$_{14}$ (0.59 g, 0.7 mmol) was dissolved in THF (20 mL) and HOBt (1.15 g, 8.51 mmol) was added into the solution, followed by stirring at 40 ^oC for 5 h. Subsequently, the obtained white solid after removal of the solvent was purified by column chromatography separation with the eluent of DCM and CH_3OH (12:1, v:v), followed by rotary evaporation. 2-(2-amino-3-(1-H-4-imidazolyl) triamino) glutaric acid ditetradecanol-diester (**H-E2C$_{14}$**), a white solid with a yield of 72.9%, was obtained after removal of organic solvents.

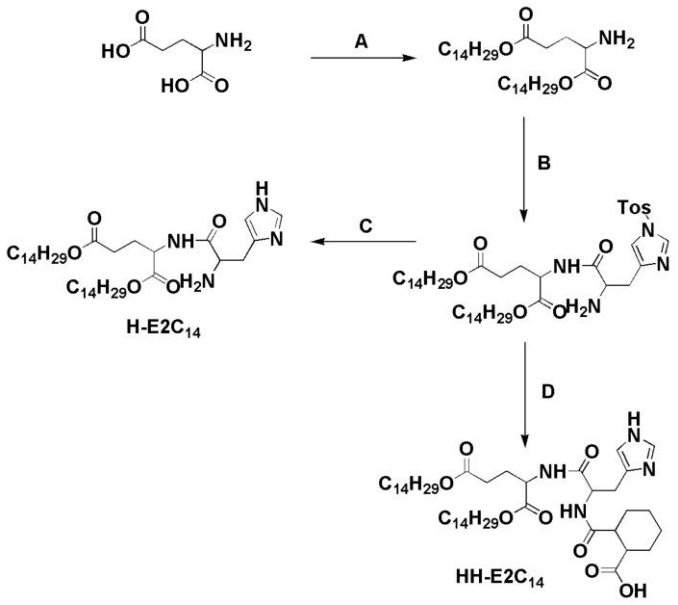
His(Tos)-E2C$_{14}$ (1.61 g, 1.9 mmol) was dissolved in DCM (40 mL). HHPA (0.30 g, 1.9 mmol) and dimethylamino pyridine (0.07 g, 0.6 mmol) were also dissolved in DCM (5 mL). The mixture of the two solutions above was stirred for 5 h. Subsequently, the obtained white solid after removal of the solvent was purified by column chromatography separation with the eluent of DCM and CH_3OH (8:1, v:v), followed by rotary evaporation. 2-(2-(2-carboxyl cyclohexylformamido)-1-(1H-imidazolyl) propionamido) glutaric acid ditetradecanol-diester (**HH-E2C$_{14}$**), a white solid with a yield of 70.1%, was obtained after removal of organic solvents.

His(Tos)-E2C$_{14}$: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.97 (2H, m), 7.35-7.83 (4H, m), 7.09 (1H, s), 4.56 (1H, m), 4.09 (4H, m), 3.66 (1H, m), 2.76-3.04 (2H, m), 2.44 (3H, s), 2.29 (1H, m), 2.18 (1H, m), 2.01 (4H, m), 1.62 (4H, m), 1.25 (42H, m), 0.88 (6H, m). HRMS, ESI^+, m/z: Calcd for

$C_{46}H_{79}N_4O_7S$ $(M+H)^+$ 831.5664; found, 831.5675; IR (film, cm^{-1}): 3396, 2923, 2853, 1736, 1666, 1511, 1468, 1376, 1191, 1174, 1078, 813, 705, 676, 592, 541.

H-E2C$_{14}$: ^1H NMR (300 MHz, $CDCl_3$) δ: 7.97 (1H, m), 7.48-7.59 (4H, m), 7.08 (1H, s), 4.61 (1H, m), 4.34 (1H, m), 3.94 (4H, m), 3.33 (1H, m), 3.18 (1H, m), 2.24 (2H, m), 1.90-2.08 (2H, m), 1.48 (4H, m), 1.22 (44H, m), 0.86 (6H, m). HRMS, ESI^+, m/z: Calcd for $C_{39}H_{73}N_4O_5$ $(M+H)^+$ 677.5575; found, 677.5575; IR (film, cm^{-1}): 3225, 2922, 2852, 2629, 1741, 1686, 1553, 1467, 1450, 1399, 1201, 1101, 742, 628, 599, 515.

HH-E2C$_{14}$: ^1H NMR (300 MHz, $CDCl_3$) δ: 7.86 (1H, m), 7.02(1H, m), 5.08 (1H, m), 4.56 (1H, m), 4.05 (4H, m), 2.81-3.00 (2H, m), 2.42 (2H, m), 2.26 (1H, m), 2.08 (3H, m), 1.79 (2H, m), 1.58 (6H, m), 1.24 (48H, m), 0.87 (6H, m). HRMS, ESI^+, m/z: Calcd for $C_{47}H_{83}N_4O_8$ $(M+H)^+$ 831.6205, found, 831.6213. IR (film, cm^{-1}): 3418, 2924, 2854, 1936, 1738, 1651, 1545, 1451, 1398, 1259, 1197, 1129, 1099, 743, 629.



Scheme S2 Synthesis of H-E2C$_{14}$ and HH-E2C$_{14}$. Reagents and reaction conditions: A: TsOH, $C_{14}H_{29}OH$; B: Boc-L-His(Tos)-OH, TFA; C: HOBt; D: HHPA, HOBt.

Preparation and Characterization of Liposomes.

DOX-loaded acid/redox dual-activated liposome (DOX/HH-SS-L) was prepared by an ammonium sulfate gradient method. Briefly, lipids (SPC:DOPE:HH-SS-E2C_{14}, 4:3:1, mol:mol:mol) were co-dissolved in the mixture of DCM and CH_3OH and the solvents were evaporated using a rotary evaporator. Residual solvents were removed under high vacuum. The dried lipid films were hydrated with 250 mM ammonium sulfate at pH 7.4 and dispersed using an ultrasonic cell disruptor (Life Scientz Bio-tech, China). The crude liposome was then extruded repeatedly through polycarbonate membrane filters with a pore size of 0.45 μm and 0.22 μm, followed by dialysis against 900 mL of the HEPES buffer (25 mM, pH 7.4) containing 10% sucrose and 0.1 mM EDTA overnight. The blank liposome was incubated with the HEPES buffer (25 mM, pH 7.9) containing 10% sucrose and DOX at a DOX/lipid ratio of 1:20 (w:w) under gentle shaking at room temperature for 1 h. Non-encapsulated DOX was separated from the liposome by passing over Sephadex G-50 column. The obtained DOX/HH-SS-L was stored in dark at 4 ^oC for later use. Other kinds of DOX-loaded synthetic liposomes, such as DOX/H-L, DOX/HH-L and DOX/H-SS-L, were prepared using the same method with DOX/HH-SS-L, except that the functionalized lipid, HH-SS-E2C_{14} was replaced with H-E2C_{14}, HH-E2C_{14} and H-SS-E2C_{14}, respectively, while DOX-loaded conventional liposome (DOX/SPC-L) contained the lipid components of SPC:DOPE (2:1, mol:mol).

The encapsulation efficiency (EE) ($W_1/W_2 \times 100\%$) was calculated, where W_1 and W_2 are the quantities of DOX in the liposome after and before processed by Sephadex G-50 column, respectively. The drug-loading capacity (DL) ($W_3/W_4 \times 100\%$) was determined, where W_3 and W_4 are the quantities of DOX and lipids in the freeze-dried liposomes, respectively.

The amount of DOX was determined using HPLC. The HPLC system comprised of an LC-20AB pump, RF-10AXL fluorescence detector, SIL-20AC auto sampler and CTO-20A column

oven (SHIMADZU, Japan). An Inert Sustain® C18 column (250 mm × 4.6 mm × 5 μm) (GL Sciences Inc., Japan) was employed for the separation of analyte at a flow rate of 1 mL/min. The column temperature was 40 °C. The excitation and emission wavelength were 496 nm and 553 nm, respectively. The mobile phase was composed of methanol, water and acetic acid at 60:40:2 (v:v:v).

The mean particle diameter, polydispersity index (PDI) and zeta potential of liposomes were measured using a Dynamic Light Scattering Analyzer and a ZetaPlus Zeta Potential Analyzer (Brookhaven, USA) after dilution with specified solutions at certain times, respectively.

Acid-Responsive Charge Conversion.

To investigate acid-triggered charge conversion of the functionalized liposomes, change in zeta potential of the liposomes was determined at different pH. Briefly, 200 μL of SPC-L, HH-L or HH-SS-L was diluted in 2.2 mL of the HEPES (20 mM, pH 7.4) and acetate buffer (20 mM, pH 6.5, 5.5, 4.5) or RPMI 1640 culture medium (pH 7.4, 6.5, 5.5, 4.5) containing 50% (v:v) FBS, respectively. The zeta potential of the liposome was immediately tested at 37 °C. The experiments were conducted in triplicate.

Change in the particle size and zeta potential of liposome was further monitored at different pH over time. Briefly, 2 mL of HH-L or HH-SS-L was incubated with 4 mL of the HEPES buffer (20 mM, pH 7.4) or acetate buffer (20 mM, pH 6.5, 5.5, 4.5) at 37 °C. At different time intervals, 500 μL of each sample was collected and dispersed in 1.5 mL of the corresponding buffer solution. The particle size and zeta potential of the obtained sample were then measured at 37 °C. The experiments were conducted in triplicate.

pH-Dependent Degradation of Hexahydrobenzoic Amide.

The degradation of the hexahydrobenzoic amide was detected using the fluorescamine method.[2,3] After incubation of HH-SS-L with the specified buffer solution at different pH (pH 7.4, 6.5, 5.5, 4.5), the fluorescence intensity suggests the degrading degree of the hexahydribenzoic amide under different pH conditions due to the exposure of primary amine group of histidine in HH-L or HH-SS-L. Briefly, 1 mL of HH-SS-L or HH-L at lipid concentration of 3 mg/mL was mixed with 2 mL of the HEPES buffer (20 mM, pH 7.4) or acetate buffer (20 mM, pH 6.5, 5.5, 4.5). After incubating at 37 ^{o}C for specified time, 150 μL of each sample was diluted into 3 mL of CH_3OH and added with 100 μL of the fluorescamine acetone solution (2 mg/mL). After incubation in dark at room temperature for 10 min, the fluorescence intensity was determined at the excitation wavelength of 382 nm and the emission wavelength of 474 nm by fluorospectrophotometer (RF-5301 PC, SHIMADZU, Japan). 100% of exposed amine was calculated from the fluorescence intensity of the sample after the incubation of HH-L or HH-SS-L in acetic acid (HAc) (20 mM) for 48 h, and 0% was from that of the fluorescence intensity of the blank buffer solution as a negative control. The experiments were conducted in triplicate.

Buffering Capacity.

To explore the buffer capacity of HH-SS-L and HH-L, acid titration was performed using a 20 mL of each liposome (5 mg/mL, lipid concentration) adjusted to pH 10 with 0.3 M NaOH. Successive additions of the HCl solution (0.01 M) were then conducted, followed by the pH measurement.

Kinetics of Disulfide Cleavage.

To assess the kinetics of disulfide degradation, the remained H-SS-E2C_{14} in H-SS-L was quantitatively analyzed by HPLC after incubation with different concentrations of glutathione (GSH)

over time. Typically, 10 mL of H-SS-L (5 mg/mL, lipid concentration) was incubated in the presence of different GSH levels (0, 0.01, 1, 10 mM) at pH 7.4 at 37 ^{o}C. At specified time intervals, 1 mL of solution was sampled and mixed with 1 mL of methanol, followed by 2 min of vortex. Afterward, 4 mL of $CHCl_3$ was added and vortexed for 5 min. 0.5 mL of the saturated NaCl solution was slowly added for demulsification. The samples were centrifuged at 10000 g for 10 min at 4 ^{o}C。The subnatant was harvested, evaporated under high vacuum at 30 ^{o}C and reconstituted in 200 μL of mobile phase (acetonitrile: methanol: dichloromethane: diethylamine, 40:30:30:0.2, v:v:v:v). The sample was then vortexed for 5 min and centrifuged at 10000 g for 10 min at 4 ^{o}C. 50 μL of the supernatant was injected into and analyzed by HPLC at the wavelength of 291 nm. The retention time was about 5.5 min. The total quantity of H-SS-E2C_{14} in H-SS-L with the GSH treatment was determined according to the same process. The experiments were conducted in triplicate.

Reduction-Triggered Drug Release.

The kinetics of *in vitro* DOX release from different DOX-loaded liposomes were determined at different conditions using the dialysis method. Briefly, 0.5 mL of different DOX-loaded liposomes were added into a dialysis bag (MWCO 14000), followed by immersing into 30 mL of different buffer solution and incubating under the aerated nitrogen condition at 50 rpm and 37 ^{o}C At predesigned time intervals, 1 mL of release media was sampled and replenished with an equal volume of fresh media. The amount of DOX released was detected using HPLC. The experiments were conducted in triplicate. In addition, to investigate the intracellular GSH-triggered disruption of DOX/HH-SS-L, the transmission electron microscope (TEM) examination was applied. Briefly, 2 mL of DOX/HH-SS-L was incubated with 10 mM GSH at 37 ^{o}C for 24 h. Afterward, a drop of

solution was placed on a carbon-coated copper grid (100 mesh) and allowed to stand for 1 min. After removal the excessive solution, the sample was stained with the aqueous phosphotungstic acid solution (2%) for 1 min. The obtained sample was allowed to air dry for 10 min, and then observed using TEM (H-7600, Hitachi) at the acceleration voltage of 80 kV. The untreated DOX/HH-SS-L was taken as a control.

Cell Culture.

The murine macrophages (RAW264.7) and human hepatic carcinoma (HepG2) cells (Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI 1640 with 10% (v:v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in a cell incubator (Thermo Scientific, USA) at 37 ^{o}C under an atmosphere of 5% CO_2 and 90% relative humidity.

Cellular Uptake and Endocytic Pathway.

HepG2 cells (1×10^5 cells/well) were seeded in 24-well plates and cultured for 48 h. Different DOX-loaded liposomes (DOX/SPC-L, DOX/HH-L, DOX/HH-SS-L) were diluted in the FBS-free culture medium (pH 7.4 or pH 6.5) to DOX concentration of 5 μg/mL. After cell incubation with the liposomes at 37 ^{o}C for 2 h, the cells were washed by ice-cold PBS thrice. The amount of DOX was determined by HPLC as follows: the cells were disrupted with 200 μL of cell lysis buffer (Beyotime, China) to release DOX in the cells. The cell lysate was harvested and centrifuged at 10000 g for 5 min. The amount of cell protein in the supernatant was quantified using the BCA protein assay kit (Beyotime, China). On the other hand, 50 μL of supernatant was mixed with 200 μL of methanol, vortexed for 5 min, and centrifuged at 10000 g for 10 min. The amount of DOX in the supernatant was determined using HPLC.

To evaluate the endocytic pathway of the liposomes, HepG2 cells were first incubated with different specific inhibitors for a variety of endocytic pathways at 37 ^{o}C for 1 h, such as amiloride (133 μg/mL) (a macropinocytosis inhibitor), sucrose (154 μg/mL) (a clathrin-mediated endocytosis inhibitor) and nystatin (15 μg/mL) (a caveolin-mediated endocytosis inhibitor).[4-6] Afterward, different DOX-loaded liposomes were added at a DOX concentration of 5 μg/mL, followed by an additional 2 h of incubation at 37 ^{o}C. The solution was then removed, and the cells were washed by ice-cold PBS thrice. The amount of DOX was determined by HPLC. Uptake of DOX ($Q_{DOX}/Q_{cell\ protein}$) was calculated, where Q_{DOX} and $Q_{cell\ protein}$ are the quantities of DOX and cell protein, respectively. Compared with the uptake in the absence of the inhibitors, the significantly decreasing relative uptake of the DOX-loaded liposomes in the presence of the inhibitors indicates the corresponding endocytic pathways of the liposomes.

To investigate the uptake specificity of DOX-loaded liposomes by the macrophages, RAW264.7 cells (1×10^5 cells/well) were seeded in 24-well plates and cultured for 48 h. Different DOX-loaded liposomes (DOX/SPC-L, DOX/HH-L and DOX/HH-SS-L) were diluted in the FBS-free culture medium (pH 7.4) to DOX concentration of 5 μg/mL. After cell incubation with the liposomes at 37 ^{o}C, the cells were washed by ice-cold PBS thrice. The following procedures were the same with the above mentioned. The cellular uptake of different DOX liposomes was determined.

Endo-Lysosomal Escape and Intracellular Release.

The subcellular localization and intracellular release behavior of the DOX-loaded liposomes in cancer cells were visualized using confocal laser scanning microscope (CLSM). HepG2 cells (5×10^4 cells/well) were cultured in a confocal dish (Greiner Bio-One, Germany) at 37 ^{o}C for 24 h,

followed by incubation with different DOX-loaded liposomes (DOX/SPC-L, DOX/HH-L, DOX/HH-SS-L) at a DOX concentration of 5 μg/mL at pH 6.5. After 2 h of incubation, the excessive liposomes were removed. The cells were washed by ice-cold PBS thrice and incubated with the FBS-free culture medium for another 1 h. Afterward, the cells were washed by ice-cold PBS thrice. The late endosome and lysosome were stained with 50 nM LysoTracker Green at 37 ^{o}C for 1 h. The cells after treated for 2 h or 2 h plus 1 h (2 h + 1 h) were observed using CLSM (TCS-SP5, Leica, Germany).

Cell Apoptosis.

The apoptosis-inducing capabilities of the DOX-loaded liposomes on cancer cells were evaluated using the Annexin V-FITC/PI double staining method. Briefly, HepG2 cells (1×10^6 cells/well) were seeded in 6-well plates for 48 h at 37 ^{o}C, and then incubated with different DOX-loaded liposomes (DOX/SPC-L, DOX/HH-L, DOX/HH-SS-L) at a DOX concentration of 200 ng/mL at pH 6.5 for 2 h, respectively. The cells were then washed by ice-cold PBS thrice, and incubated with the FBS-free culture medium for another 4 h. Afterward, the cells were harvested, washed by ice-cold PBS twice, and suspended in the binding buffer. 5 μL of Annexin V-FITC was added into the cell suspensions for 15 min of incubation, and then 5 μL of propidium iodide (PI) was added. The cells were immediately analyzed by flow cytometry (Becton Dickinson, USA).

Cytotoxicity Assay.

Cytotoxicity of the DOX-loaded liposomes against cancer cells was estimated using MTT assay. HepG2 cells (6×10^3 cells/well) were seeded in 96-well plates and cultured for 24 h. The cells were exposed to the FBS-free culture medium containing different DOX-loaded liposomes (DOX/SPC-L,

DOX/HH-L, DOX/HH-SS-L) at a variety of DOX concentrations for 6 h at pH 6.5. The cells were then washed by ice-cold PBS thrice, and incubated with 200 μL of the FBS-free culture medium for another 42 h or 66 h. 20 μL of the MTT solution (5 mg/mL) was then added into each cell, followed by 4 h of incubation. The medium was removed, and the cells were mixed with 150 μL of DMSO. The absorbance was determined at the wavelength of 570 nm using a microplate reader (Thermo Scientific, USA).

Animals and Tumor Xenograft Models.

Institute of Cancer Research (ICR) mice (male, 25-30 g) and Sprague-Dawley (SD) rats (male, 180-220 g) were purchased from College of Veterinary Medicine Yangzhou University (Jiangsu, China). All the animals were pathogen free and allowed to access food and water freely. The experiments were conducted in compliance with the Guide for Care and Use of Laboratory Animals, approved by China Pharmaceutical University. The tumor-bearing mice were constructed by injecting 100 μL of PBS containing hepatocellular carcinoma (Heps) cells (1×10^6 cells/mouse) into the subcutaneous dorsa of mice. Tumor volume ($V = L \times W^2/2$) was determined, where L and W are the length and width of the tumor, respectively.

***In vivo* Imaging.**

The tumor targetability of the liposomes after intravenous injection was explored using the non-invasive fluorescent imaging technique. The liposomes were first stained with DiR by dissolving the lipid components with DiR during the liposome preparation. The particle sizes of the obtained different DiR-labeled liposomes (DiR/SPC-L, DiR/HH-L, DiR/HH-SS-L) were 97, 115 and 124 nm, respectively. When the tumor size reached to 0.1-0.15 cm^3, the Heps tumor-bearing

mice were intravenously injected with DiR/SPC-L, DiR/HH-L and DiR/HH-SS-L at a DiR dose of 5 mg/kg.[7] At pre-set time points, the anesthetized mice were put into the chamber and the fluorescent images were taken using the Maestro *in vivo* optical imaging system (CRi, Inc., USA) with an excitation bandpass filter at 748 nm and an emission at 780 nm. Once the completion of living imaging, the mice were sacrificed. The tumor and main normal organs including heart, liver, spleen, lung and kidney were harvested for *ex vivo* imaging using the same system mentioned above. The region-of-interest (ROI) was circle around the harvested tissues, and the fluorescence intensity of DiR was determined using the Maestro 3 software.

***In vivo* Biodistribution**

When the tumor size reached to 0.1-0.15 cm^3, the Heps tumor-bearing mice were divided randomly into four groups, and intravenously injected with the free DOX solution or different DOX-loaded liposomes (DOX/SPC-L, DOX/HH-L and DOX/HH-SS-L) at a DOX dose of 5 mg/kg, respectively. The blood samples were collected at 0.17, 1, 2, 4, 8, 12, 24 and 48 h after injection, and then centrifuged at 10000 × g for 10 min. The tumor and main organs including heart, liver, spleen, lung, and kidney were collected and weighed. To quantify the DOX amount in the plasma and tissues, the tissues were first homogenized with saline. DOX was then extracted by mixing acetonitrile:methanol (2:1, v:v) containing 2% (v:v) concentrated HCl with the plasma or the tissue homogenate, followed by 2 min of vortex. After centrifugation at 10000 × g for 10 min, the DOX concentration in the supernatant was determined using HPLC.

Antitumor Efficacy.

When the tumor size reached to 0.1-0.15 cm^3, the Heps tumor-bearing mice were intravenously

injected with different DOX-loaded liposomes at a DOX dose of 5 mg/kg at Day 0, 4, 8 and 12. The dimension of the tumor was measured by a fine caliper and meanwhile, the total body weight was weighed. At Day 16, the mice were sacrificed. The tumors were harvested and sectioned for histological evaluation using the hematoxylin and eosin (HE) staining.

Statistical Analysis.

Data are given as mean ± standard deviation (S.D.). Statistical significance was tested by two-tailed Student's t-test or one-way ANOVA. Statistical significance was set at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, respectively.

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Table S1. Particle size, zeta potential, EE and DL of the optimized liposomes at the HEPES buffer (20 mM, pH 7.4) (Mean ± S.D., *n* = 3).

Liposomes	Size (nm)	PDI	Zeta potential (mV)	EE (%)	DL (%)
DOX/SPC-L	119 ± 2	0.119 ± 0.002	-10 ± 1	97.6 ± 2.7	3.9 ± 0.7
DOX/HH-L	126 ± 5	0.182 ± 0.012	-16 ± 2	92.5 ± 2.3	3.1 ± 1.0
DOX/HH-SS-L	131 ± 2	0.194 ± 0.015	-18 ± 2	95.9 ± 6.0	3.1 ± 0.7
DOX/H-L	95 ± 5	0.121 ± 0.014	+33 ± 4	92.6 ± 3.7	3.2 ± 0.5
DOX/H-SS-L	99 ± 3	0.105 ± 0.009	+30 ± 4	93.3 ± 2.5	3.2 ± 0.3

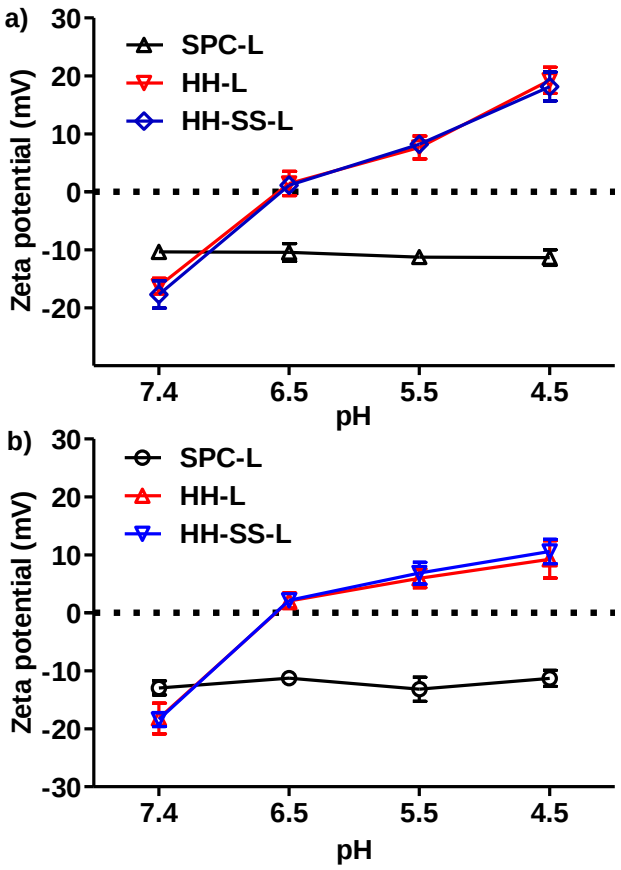

Fig. S1. Zeta potentials of SPC-L, HH-L and HH-SS-L in the buffer solutions (a) and in the RPMI 1640 culture medium containing 50% (v:v) FBS (b) at different pH values.

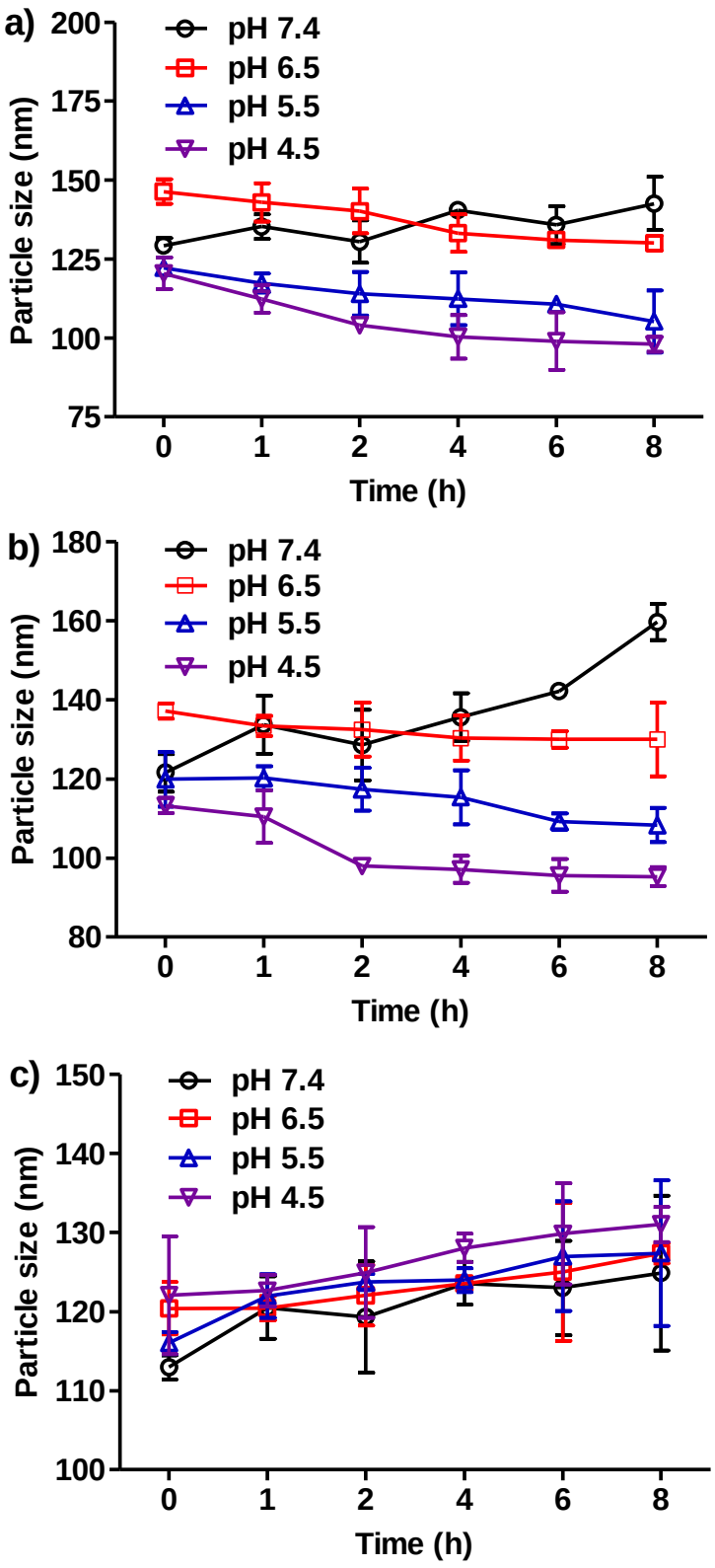

Fig. S2. Average particle sizes of HH-SS-L (a), HH-L (b) and SPC-L (c) at different pH values.

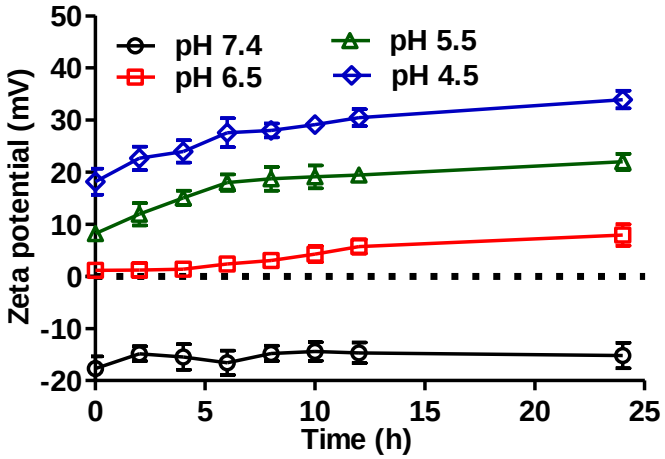

Fig. S3. Zeta potential variation of HH-SS-L at different pH values.

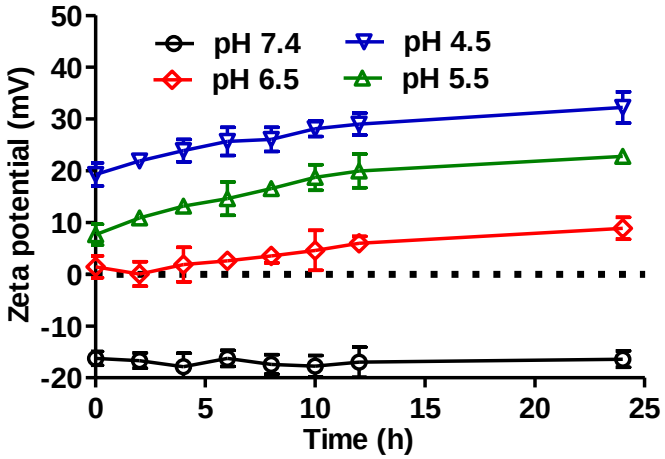

Fig. S4. Zeta potential variation of HH-L at different pH values.

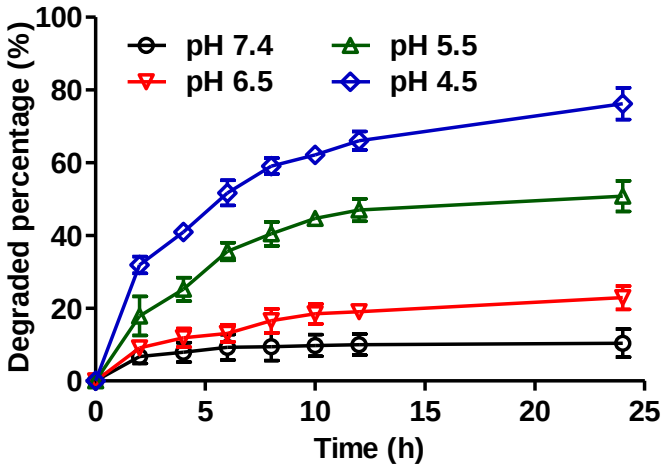

Fig. S5. Degradation of the HHB amide in HH-L at different pH values.

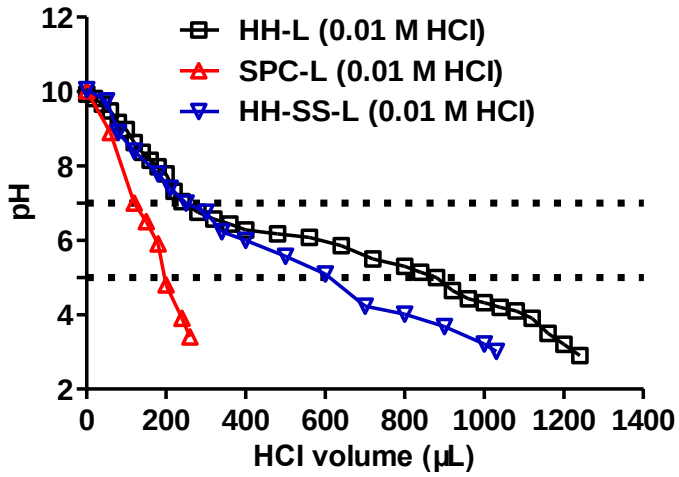

Fig. S6. Acid titration profiles of SPC-L, HH-L and HH-SS-L. Liposomes were adjusted to pH 10 with 0.3 M NaOH solution and then titrated by 0.01 M HCl.

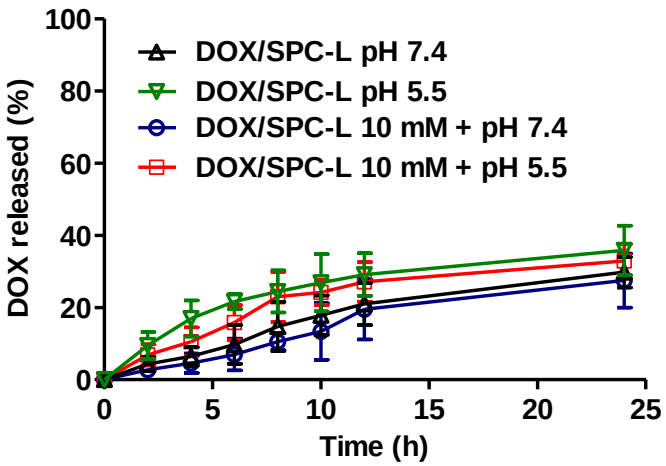

Fig. S7. *In vitro* release profiles of DOX from DOX/SPC-L under different conditions.

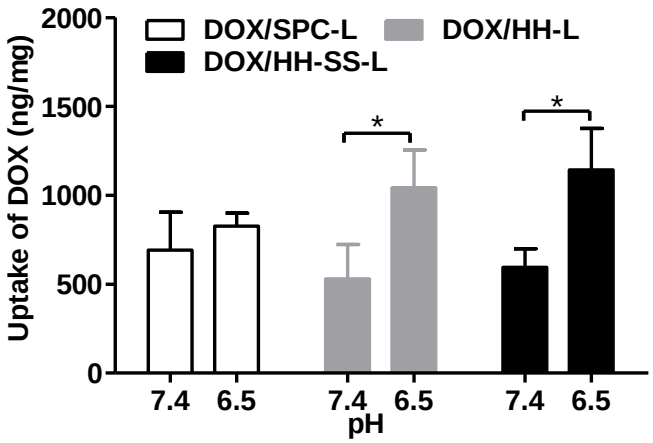

Fig. S8. Cellular uptake of DOX/SPC-L, DOX/HH-L and DOX/HH-SS-L on HepG2 cells at different pH values. $^{*}P < 0.05$.

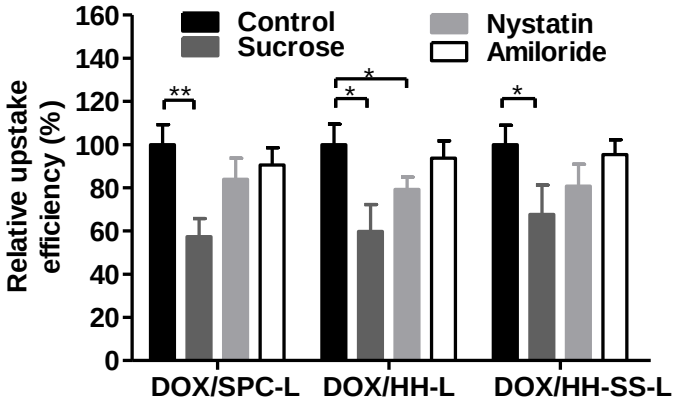

Fig. S9. Relative uptake efficiency of DOX/SPC-L, DOX/HH-L and DOX/HH-SS-L on HepG2 cells in the presence of various endocytosis inhibitors. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control group. Sucrose, nystatin and amiloride are the inhibitors of clathrin-mediated endocytosis, caveolin-mediated endocytosis and macropinocytosis, respectively. Compared with the cellular uptake of the DOX-loaded liposomes in the absence of inhibitors as a control, the significant reduction in the cellular uptake of the DOX-loaded liposomes in the presence of inhibitors indicated the corresponding endocytic pathways of the liposomes.

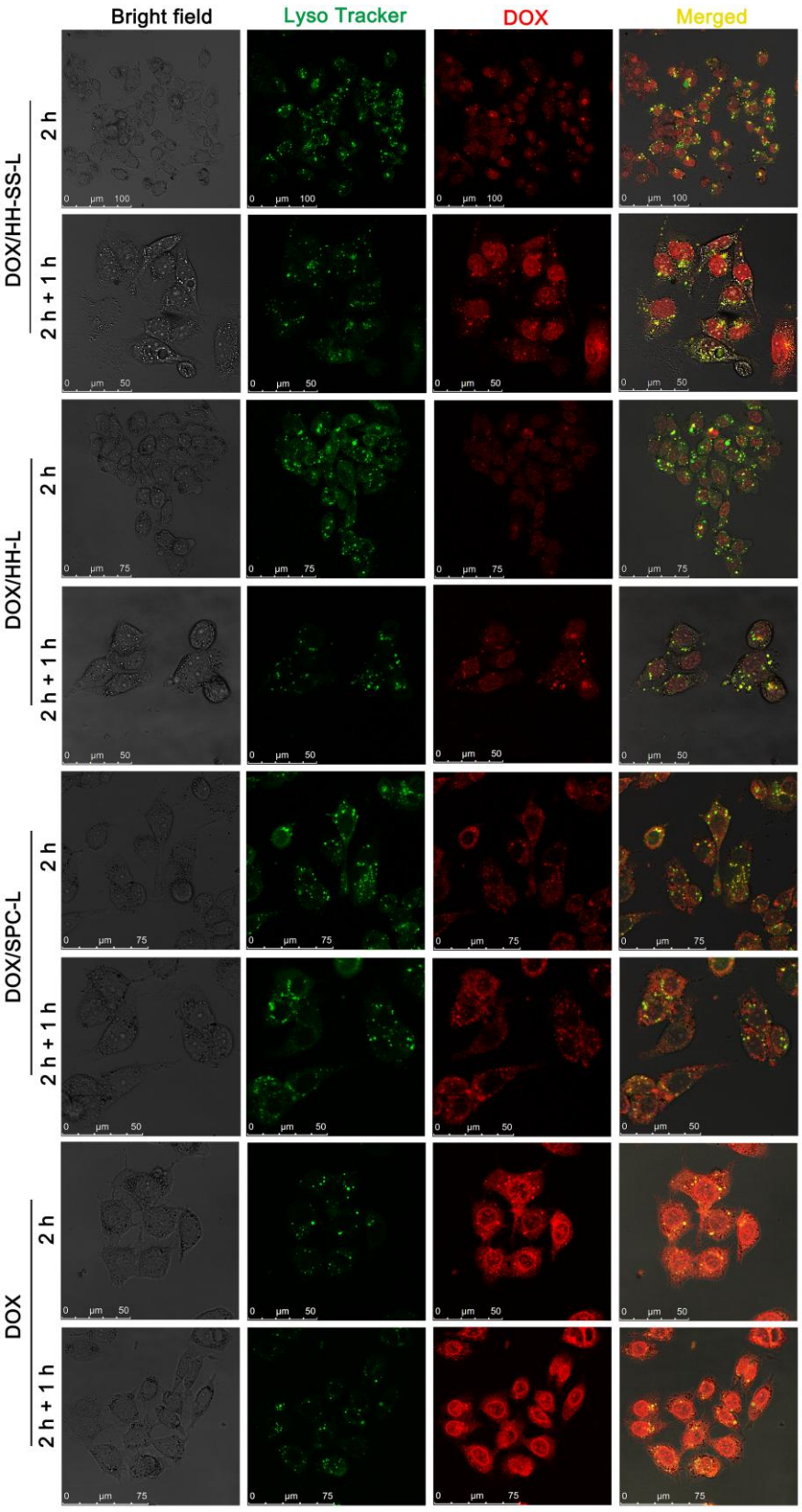

Fig. S10. CLSM images of HepG2 cells after incubation with free DOX, DOX/SPC-L, DOX/HH-L and DOX/HH-SS-L for different time. The late endosomes and lysosomes were stained with LysoTracker Green.

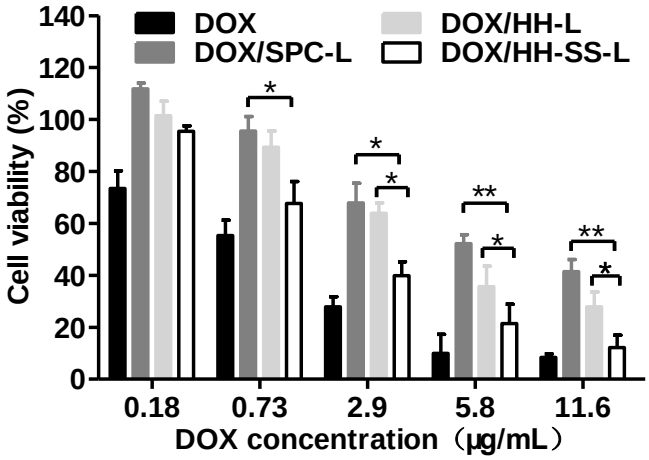

Fig. S11. *In vitro* cytotoxicity of the free DOX, DOX/SPC-L, DOX/HH-L and DOX/HH-SS-L against HepG2 cells for 48 h. $^{*}P < 0.05$, $^{**}P < 0.01$.

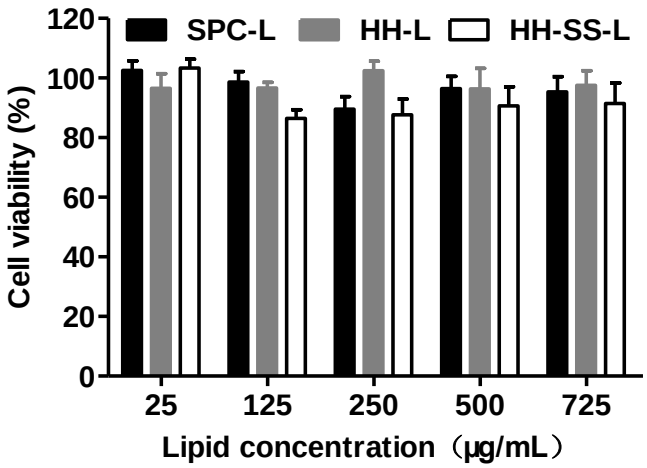

Fig. S12. *In vitro* cytotoxicity of the bare liposomes without DOX against HepG2 cells at pH 6.5 for 72 h.

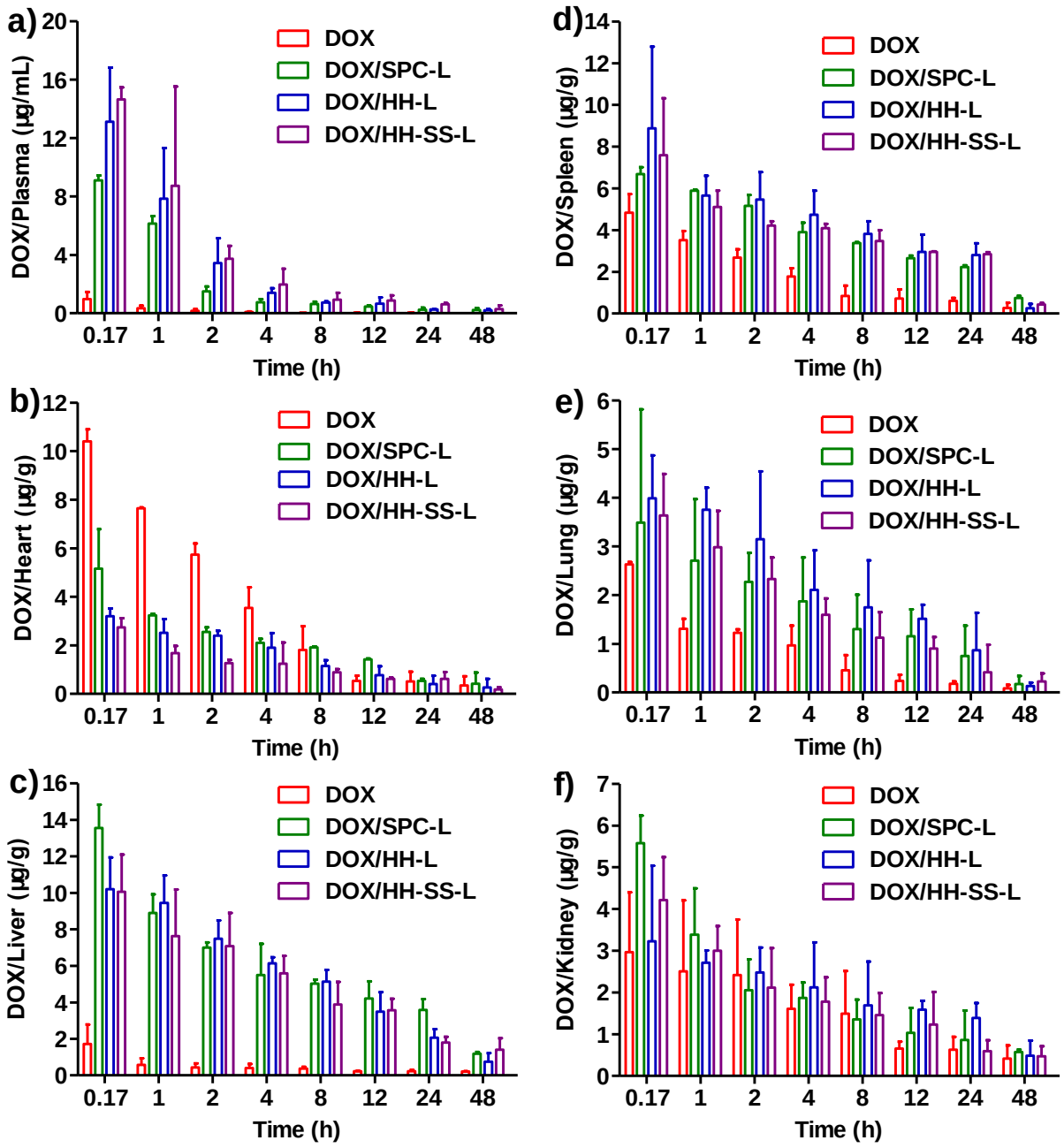

Fig. S13. Quantification on the accumulation of DOX in the plasma (a) and main organs of the tumor-bearing mice, including heart (b), liver (c), spleen (d), lung (e), kidney (f), after intravenous injection of different DOX formulations at a DOX dose of 5 mg/kg. DOX/Tissue is the ratio of the DOX amount in the tissue (μg) to the tissue weight (g).

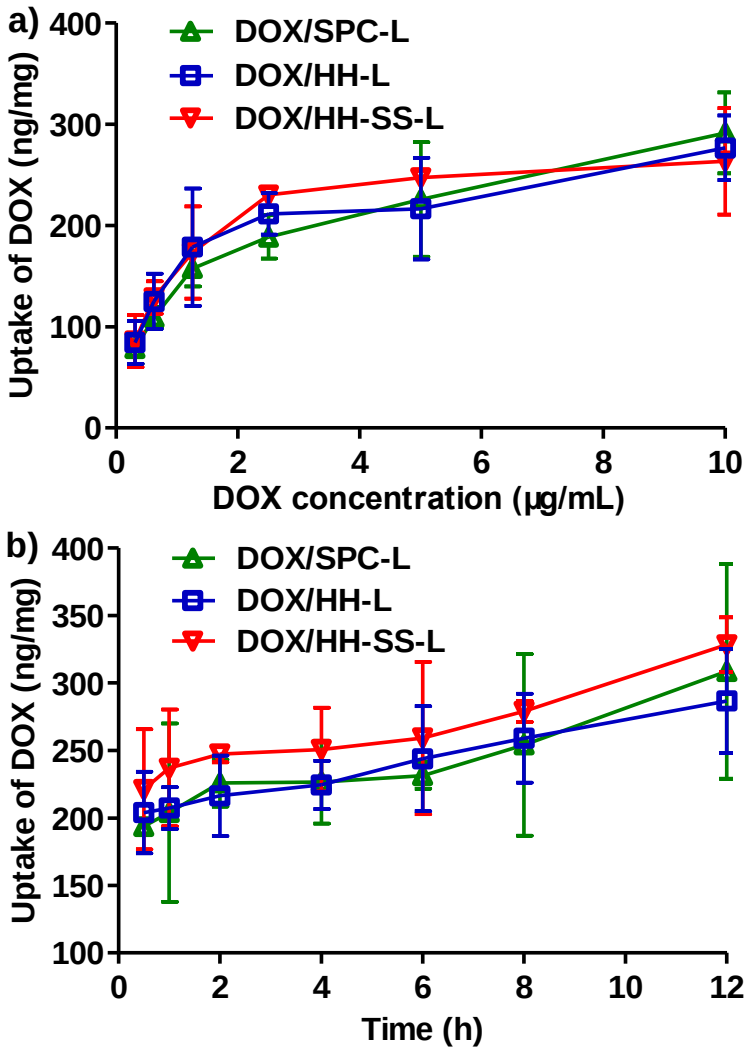

Fig. S14. Cellular uptake of DOX/SPC-L, DOX/HH-L and DOX/HH-SS-L on RAW264.7 cells at different DOX concentration after 2 h incubation (a) or at DOX concentration of 5 μg/mL for different incubation time (b).

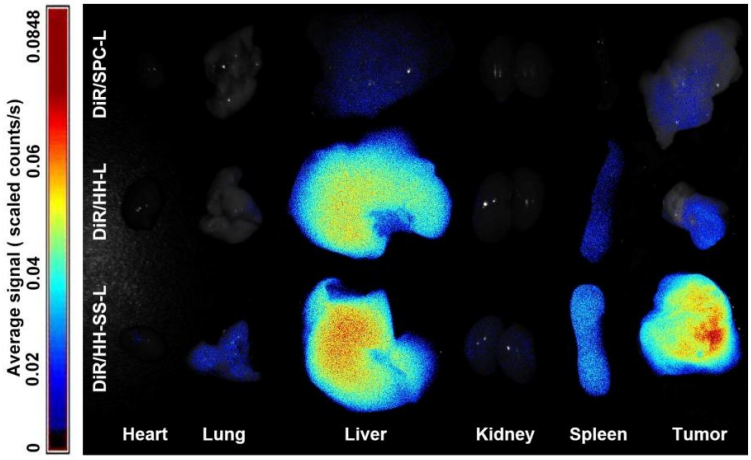

Fig. S15. *Ex vivo* fluorescence images of different tissues obtained from the xenograft Heps tumor-bearing mice at 24 h post-injection of DiR/SPC-L, DiR/HH-L and DiR/HH-SS-L.

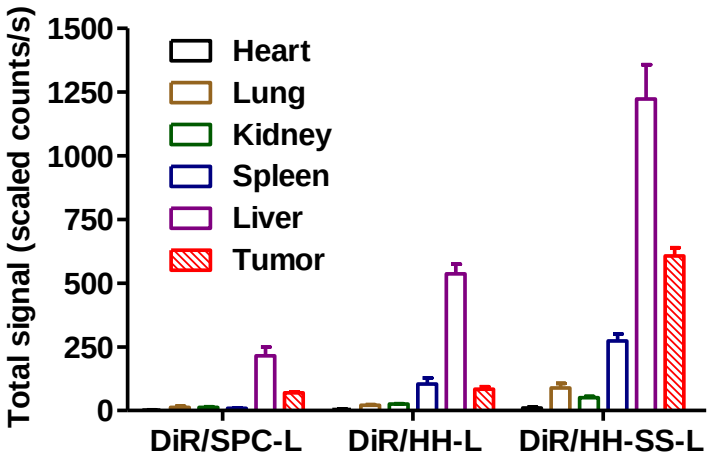

Fig. S16. Fluorescence intensity of the DiR signal in different tissues obtained from the xenograft Heps tumor-bearing mice at 24 h post-injection of DiR/SPC-L, DiR/HH-L and DiR/HH-SS-L using the *ex vivo* qualitative ROI analysis.

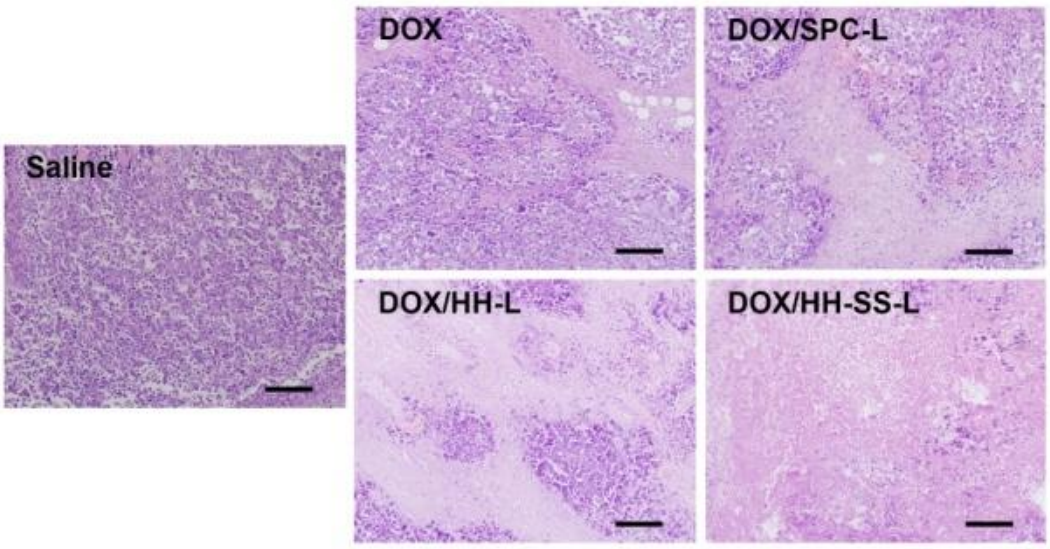

Fig. S17. Representative images of the tumor tissues stained with HE after treatment with different DOX formulations. Scale bars are 100 μm.

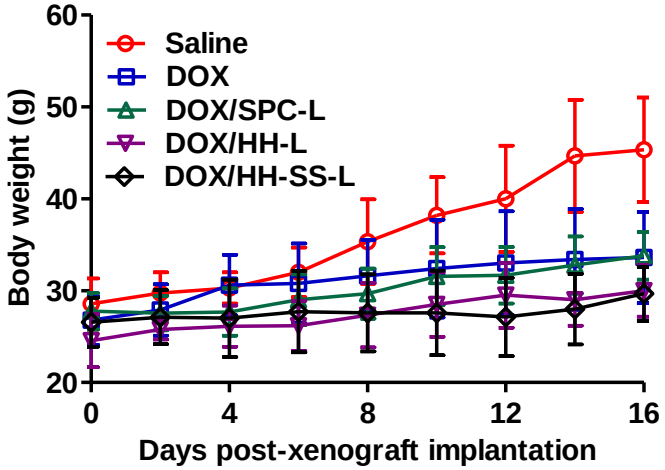

Fig. S18. Change in the body weight of the mice during the treatment with different DOX formulations.