Supplementary materials

Doxorubicin-bound hydroxyethyl starch conjugate nanoparticles with pH/redox responsive linkage for enhancing antitumor therapy

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Synthesis of 3-(2-pyridyldithio) propionic acid (PDP)

PDP was synthesized following a reported method.[1] Briefly, Py-ss-Py (4.66 g, 45.5 mmol) was dissolved in anhydrous ethanol (200 mL), followed by addition of glacial acetic acid (5 mL). The resulting mixture was vigorously stirred at ambient temperature for 10 min, and to this mixture, MPA (2.41 g, 22.7 mmol) in 40 mL of ethanol was added dropwise. After being additionally stirred at ambient temperature for 12 h, the solvent in the mixture was removed via rotary evaporation under reduced pressure. Afterwards, the obtained oily liquid product was purified by silica gel column chromatography, and the employed eluent was composed of hexane, ethyl acetate and glacial acetic acid at a ratio of 100/10/0.1 (v/v/v) in order. The dried PDP product was weighed to be ca. 4.1g, matching with a yield of around 84%. The chemical structure of PDP was identified by using ^{1}H NMR measurement and the corresponding spectrum was presented in Figure S1.

Synthesis of HES-PDP

HES (300.0 mg, 1.67 mmol of anhydroglucose unit) was dissolved in dimethyl sulfoxide to produce a 20 mL HES solution. To this solution, PDP (0.54 g, 2.53 mmol), DCC (174.0 mg, 0.84 mmol), and DMAP (51.0 mg, 0.42 mmol) were added, and the resulting mixture was vigorously stirred at ambient temperature for 48 h. The formed N,N′-dicyclohexylurea was removed by filtration, and the filtrate was added with a mixed solvent (isopropyl alcohol/ diethyl ether = 1/1, v/v, 60 mL) to form precipitate. The obtained precipitate was collected by centrifugation (8000 rpm, 10 min), and then, redissolved in dimethyl sulfoxide, followed by dialysis against ultrapure water for 3 days. After being freeze-dried, the achieved HES-PDP product was stored at 4 ℃ for further use. The ^{1}H NMR spectrum for identifying the chemical structure of HES-PDP was shown in Figure S2.

Synthesis of MP-hyd

Methyl 3-mercaptopropionate (5g, 41.7 mmol) was first dissolved in anhydrous methanol

(75mL), and the resulting solution was added with hydrazine hydrate (6.25 g, 208.3 mmol). The mixture was then vigorously stirred at ambient temperature for 12 h in a nitrogen atmosphere, followed by removal of solvent via rotary evaporation under reduced pressure. Afterwards, the obtained crude product was introduced into a mixed solution composed of acetonitrile and ether at a ratio of 4/1 (v/v), and the mixture was then stirred for 6 h. The mixture was filtered to obtain the purified solid MP-hyd product. The dry MP-hyd product was weighed to be ca. 4.34g with an approximate yield of around 87%. ^{1}H NMR spectrum for MP-hyd was given in Figure S3.

Synthesis of HES-SS-hyd

HES-PDP (900.0 mg) was dissolved in anhydrous dimethyl sulfoxide (8 mL), followed by addition of CH_3COOH (10 μL). A MP-hyd (607.0 mg, 5.06 mmol) in anhydrous dimethyl sulfoxide (2 mL) solution was added to the HES-PDP solution. The resulting mixture was vigorously stirred at ambient temperature for 72 h in a nitrogen atmosphere. After that, the mixture was poured into a mixed solution (isopropanol/ethyl ether = 1/1, v/v) to form precipitate. The precipitate was collected by centrifugation (8000 rpm, 10 min), and then redissolved in dimethyl sulfoxide. This solution was then introduced into a membrane tube (MWCO, 3500) and dialyzed against ultrapure water for 3 days to remove impurities. After being freeze-dried, the obtained product was stored at 4 ℃ for further use. The ^{1}H NMR spectrum for HES-SS-hyd was presented in Figure S4.

Synthesis of HES-NPC

Two kinds of intermediates, HES-NPC and HES-hyd, were synthesized before the synthesis of HES-hyd-DOX. Synthesis routes for HES-NPC and HES-hyd were illustrated in Scheme S1. HES-NPC was synthesized following a method similar to that described elsewhere.[2] In brief, HES (1.0 g) was dissolved in anhydrous DMSO (20 mL), and to this HES solution, a given amount of triethylamine (TEA) (311μL, 2.25 mmol) was added. To the resulting

mixture, 4-nitrophenyl chloroformate (NPC) (227 mg, 1.12 mmol) in 10 mL of anhydrous DMSO was then added dropwise. After being additionally stirred at ambient temperature for 48 h in a nitrogen atmosphere, the reaction product was introduced into a membrane tube (MWCO, 3500) and dialyzed against ultrapure water for 3 days, followed by freeze-drying. The obtained product was stored at 4 ℃ for further use.

Synthesis of HES-hyd

HES-NPC (1.0 g) was dissolved in anhydrous DMSO (20 mL), and to this solution, a known amount of hydrazine hydrate (545 mg, 11.24 mmol) was added. The resulting mixture was vigorously stirred at ambient temperature for 24 h, and further processed by dialysis against ultrapure water for 3 days using a membrane tube (MWCO, 3500). The dry HES-hyd product was used the following synthesis of HES-hyd-DOX.

Synthesis of HES-hyd-DOX

HES-hyd (0.5g) was dissolved in anhydrous DMSO (20 mL), and to this solution, TEA (311μL, 2.25 mmol) and DOX·HCl (122 mg, 0.21 mmol) were added. The resulting mixture was vigorously stirred at ambient temperature for 48 h in a nitrogen atmosphere. Subsequently, the reaction mixture was introduced into a mixed solution (ethanol/ethyl ether = 1/1, v/v) to produce a precipitate. The precipitate was collected by centrifugation (8000 rpm, 10 min), and then redissolved in dimethyl sulfoxide. The solution was placed in a membrane tube (MWCO, 3500) and dialyzed against ultrapure water for 3 days. After being freeze-dried, the obtained HES-hyd-DOX product was stored at 4 ℃ for further use. The ^{1}H NMR spectrum for HES-hyd-DOX was given in Figure S4.

Synthesis of HES-DTDPA and HES-ODA

To synthesize HES-SS-DOX and HES-DOX, two kinds of intermediates, HES-DTDPA and HES-OD, were first prepared following the synthesis routes described in Scheme S2.

DTDPA (2.95g, 14.05 mmol) was dissolved in anhydrous DMSO (20 mL), and to this

DTDPA solution, DCC (579 mg, 2.81 mmol) and DMAP (171.0 mg, 1.41 mmol) were added. The resulting mixture was vigorously stirred at ambient temperature for 0.5 h, and then, HES (5.00 g) was added dropwise. After being additionally stirred at ambient temperature for 36 h, the formed N,N′-dicyclohexylurea was removed by filtration. The filtrate was precipitated using a mixed solvent (isopropanol/diethyl ether = 1/1, v/v). The precipitate was collected by centrifugation (8000 rpm, 10 min), and then redissolved in dimethyl sulfoxide. This solution was processed by dialysis against ultrapure water for 3 days using a membrane tube (MWCO, 3500). The freeze-dried product was stored at 4 ℃ for further use. HES-ODA was synthesized using the same method but obtained with the same reaction with HES-DTDPA.

Synthesis of HES-SS-DOX and HES-DOX

HES-SS-DOX was synthesized using a method similar to that described in our previous study.[3] HES-DTDPA (540 mg) was dissolved in anhydrous DMSO (20 mL), and to this HES-DTDPA solution, TEA (21.0 μL, 0.15mmol), EDCI (58.0 mg, 0.30 mmol), NHS (70 mg, 0.61 mmol) and DOX·HCl (88 mg, 0.15 mmol) were added. The resulting mixture was vigorously stirred at ambient temperature for 48 h. Thereafter, the mixture was poured into a given volume of methanol to form a precipitate. The precipitate was collected by centrifugation (8000 rpm, 10 min), and then, redissolved in dimethyl sulfoxide for following dialysis against ultrapure water for 3 days using a. After being freeze-dried, the obtained product was stored at 4 ℃ for further use a membrane tube (MWCO, 3500). HES-DOX was synthesized using the same method applied to the synthesis of HES-DOX. ^{1}H NMR spectra for identifying the structure of HES-SS-DOX and HES-DOX were presented in Figure S5 and S6, respectively.

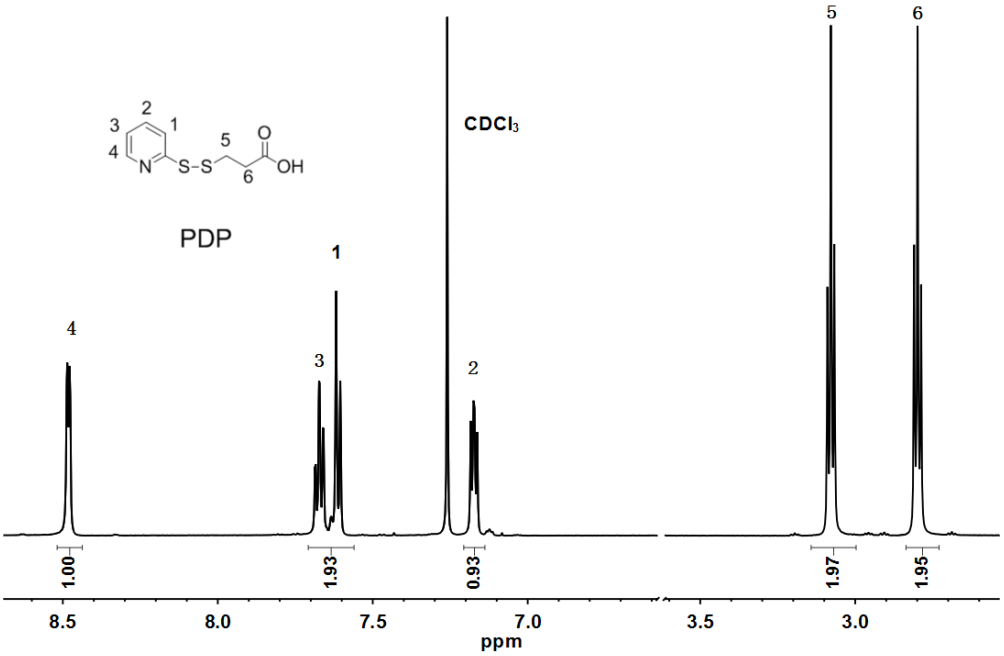


Figure S1. ^{1}H NMR spectrum of PDP.

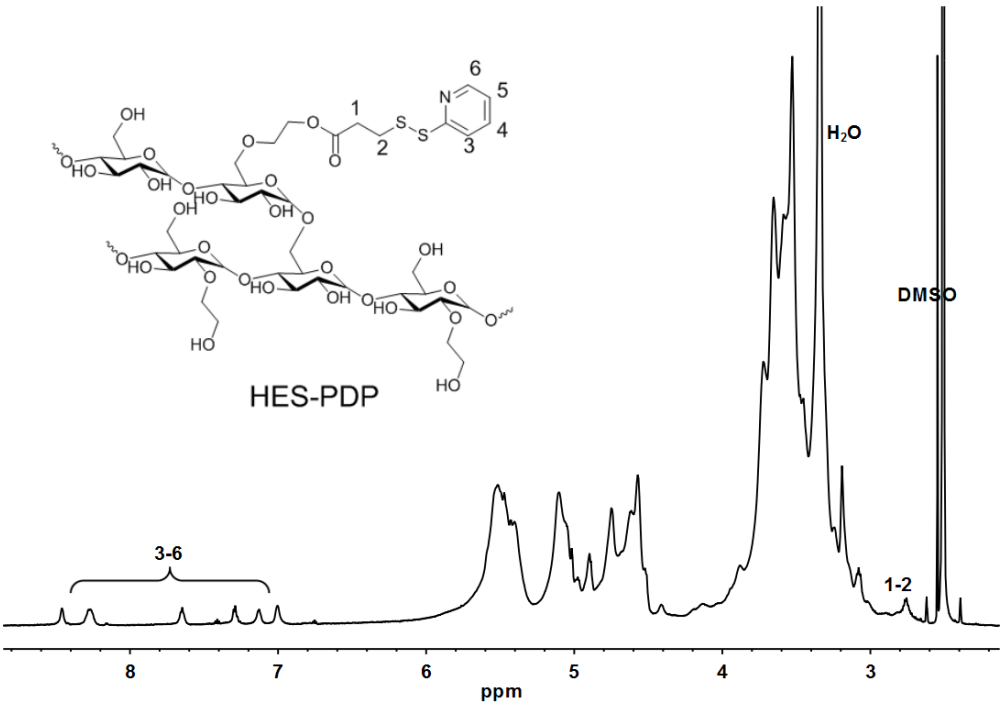


Figure S2. ^{1}H NMR spectrum of HES-PDP.

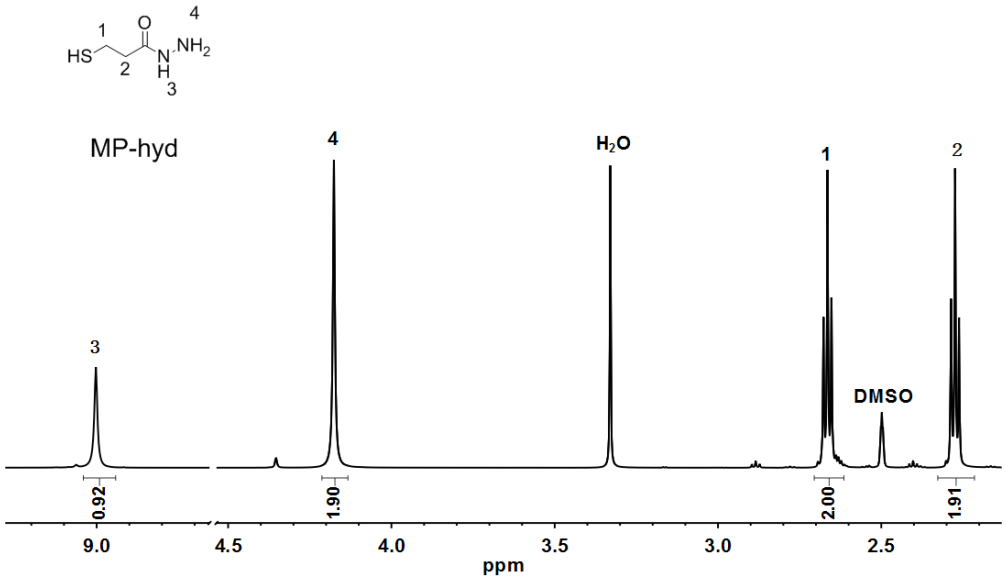
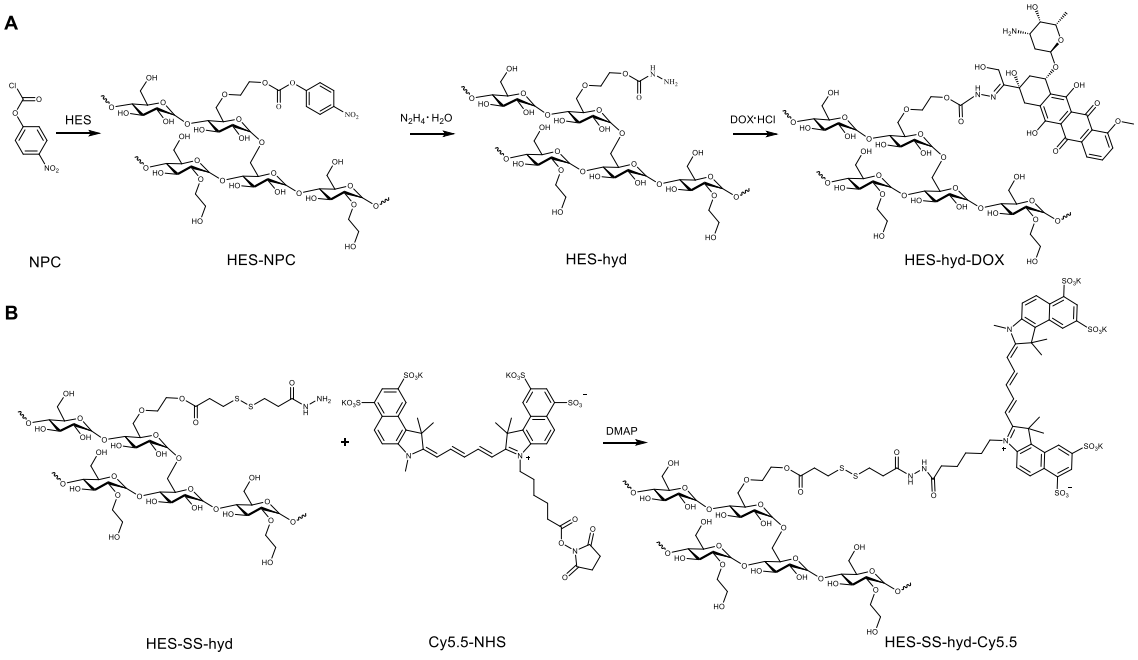


Figure S3. ^{1}H NMR spectrum of MP-hyd.



Scheme S1. Synthetic routes for HES-hyd-DOX (A) and HES-SS-hyd-Cy5.5 (B).

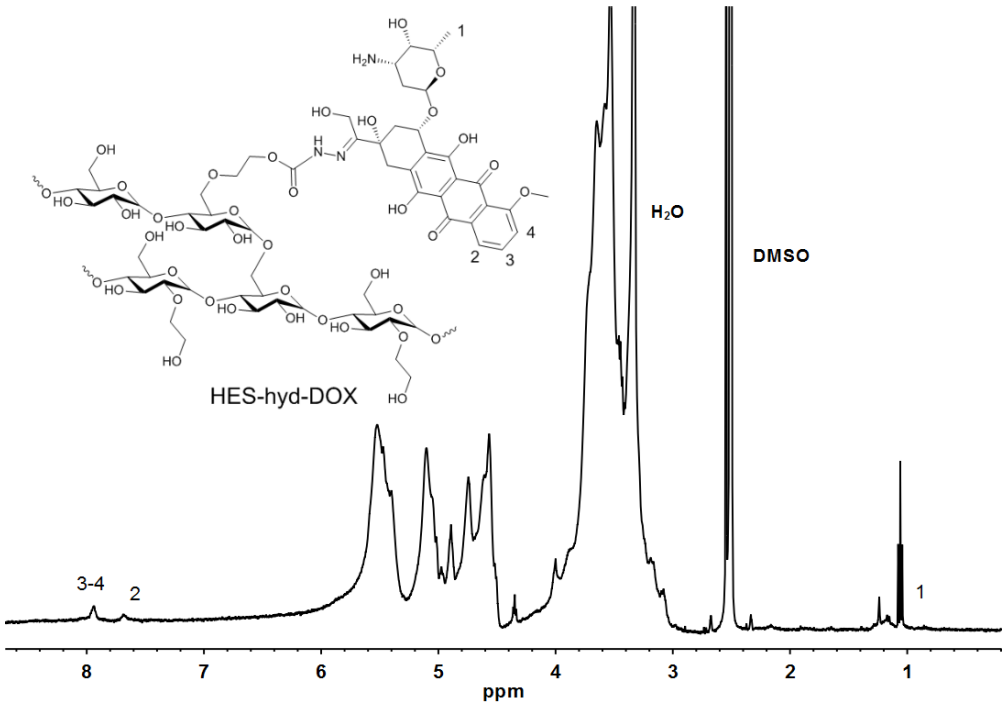
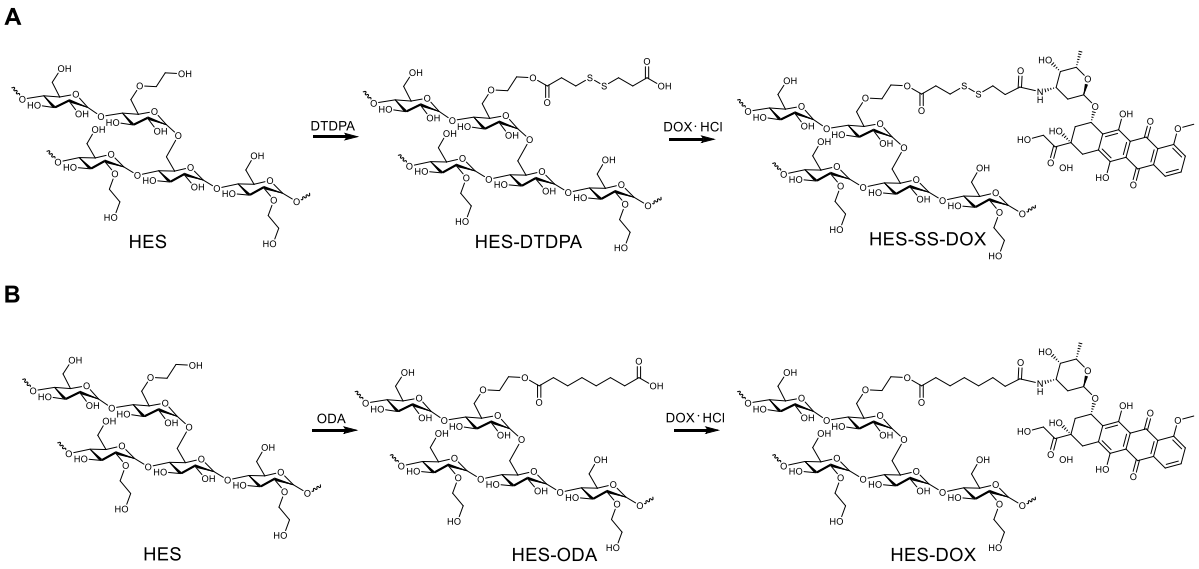


Figure S4. ^{1}H NMR spectrum of HES-hyd-DOX.



Scheme S2. Synthetic routes for HES-SS-DOX (A) and HES-DOX (B).

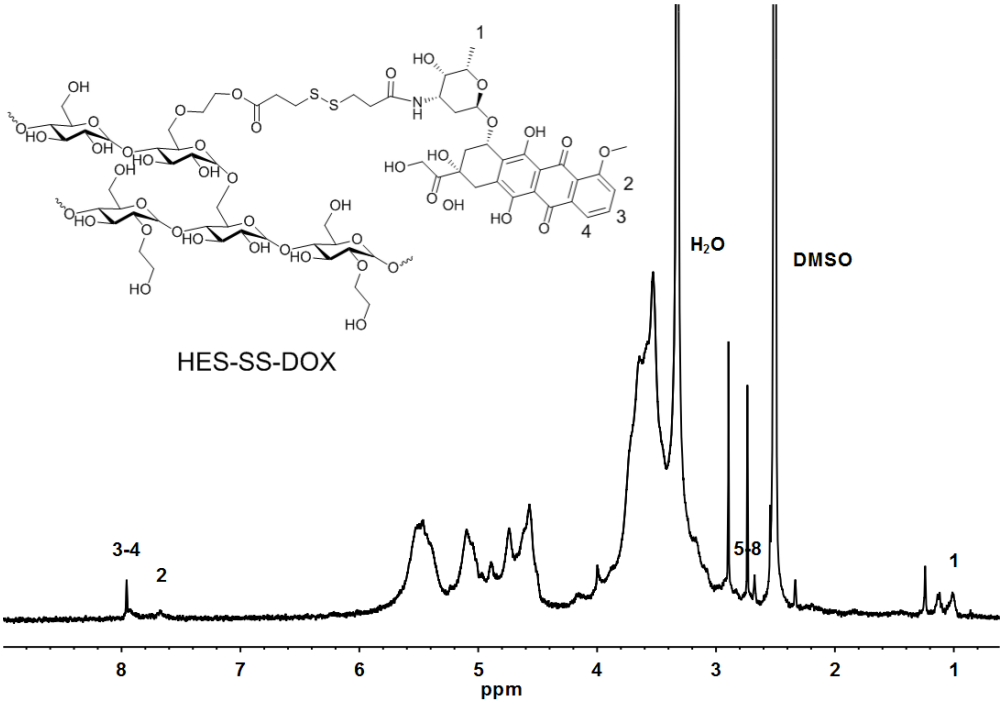


Figure S5. ^{1}H NMR spectrum of HES-SS-DOX

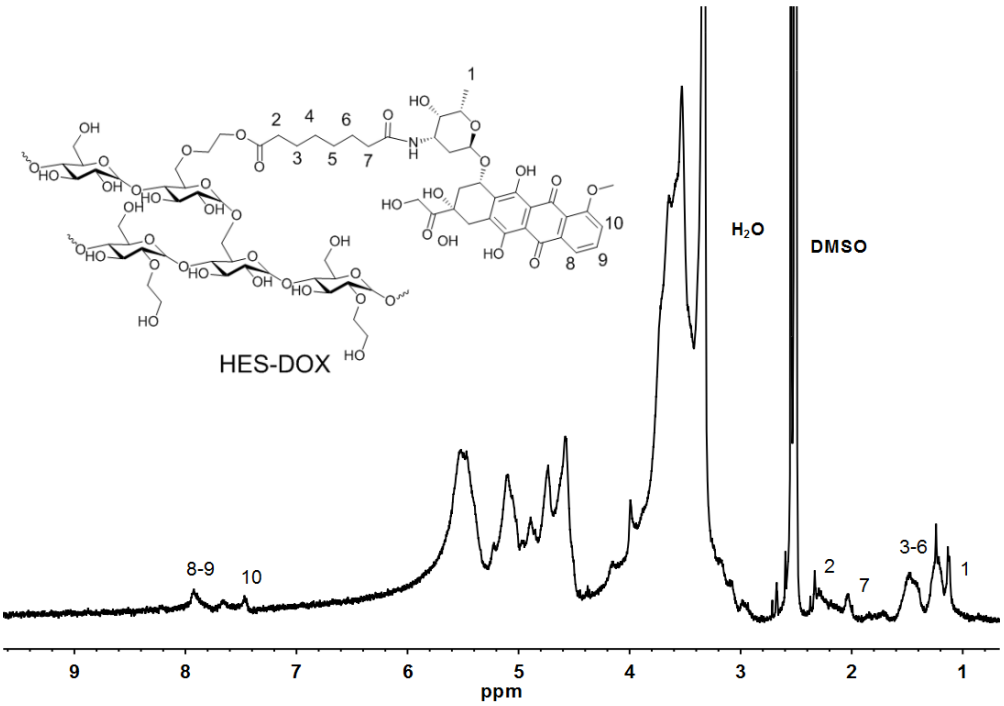


Figure S6. ^{1}H NMR spectrum of HES-DOX

Table S1 IC_{50} values for different prodrugs and free DOX[(a)]

Cell line	IC_{50} (μg/mL)				
	HES-SS-hyd-DOX	HES-hyd-DOX	HES-SS-DOX	HES-DOX	DOX
HepG2	1.332	2.936	2.632	9.459	0.3017

[(a)] Culture time: 48h

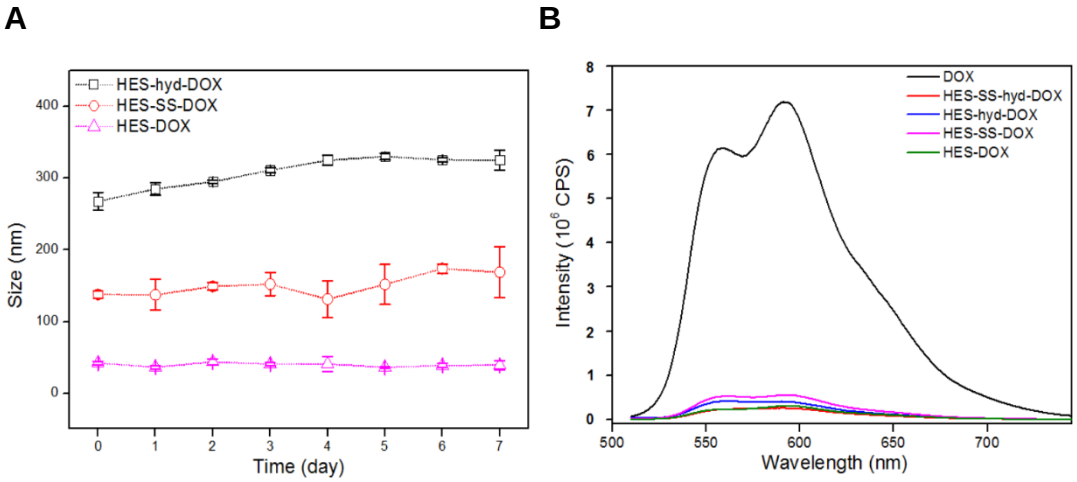


Figure S7 Size change (A) of conjugate NPs stored in PBS for various durations; and fluorescence spectra (B) for free DOX and different conjugate NPs (equivalent DOX concentration: 4 μg/mL, solvent: PBS, pH 7.4).

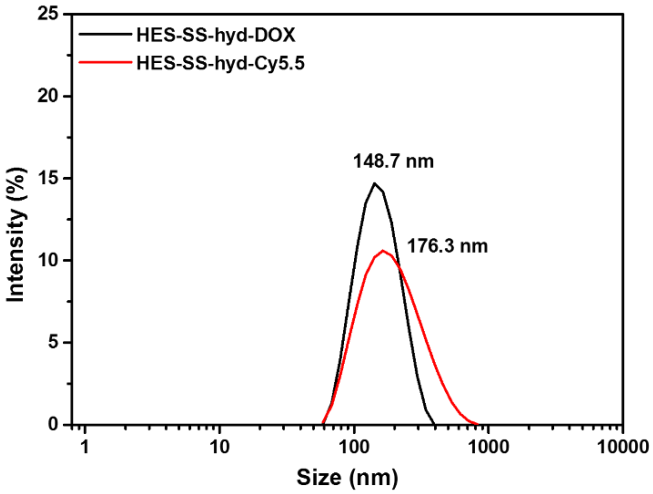


Figure S8. Size distribution of HES-SS-hyd-Cy5.5 and HES-SS-hyd-DOX NPs.

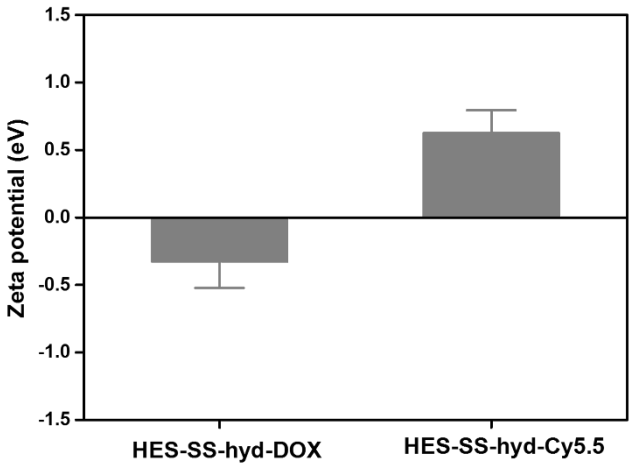


Figure S9. Zeta potential of HES-SS-hyd-Cy5.5 and HES-SS-hyd-DOX NPs.

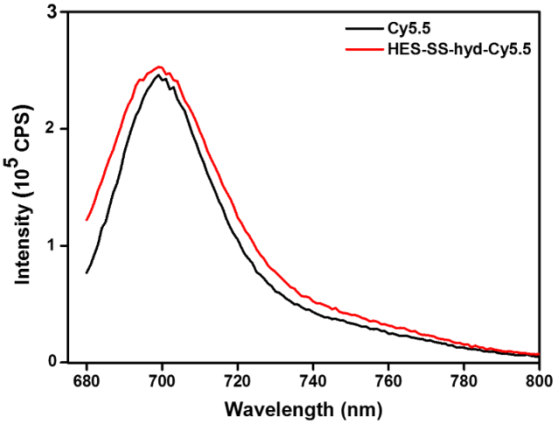


Figure S10. Fluorescence spectra of free Cy5.5 (10 μg/mL) and HES-SS-hyd-Cy5.5 (equivalent amount of Cy5.5: 10 μg/mL; solvent: PBS, pH 7.4).

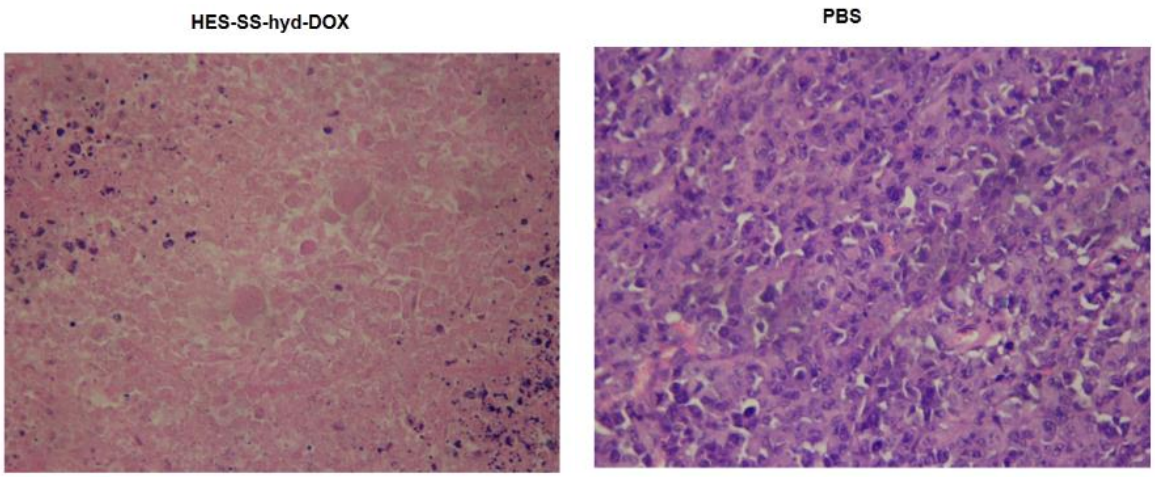


Figure S11. H/E staining micrographs with higher magnification (400×) for the tumor tissue excised from H22-tumor-bearing mice treated with HES-SS-hyd-DOX and PBS (equivalent DOX dose for HES-SS-hyd-DOX group, 4 mg/(kg of body weight)).

References

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3. Hu H, Li YH, Zhou Q, et al. Redox-sensitive hydroxyethyl starch-doxorubicin conjugate for tumor targeted drug delivery. *ACS Appl Mater Interfaces.* 2016; 8: 30833-30844. doi: 10.1021/acsami.6b11932