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Electronic Supplementary Information (ESI)

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pH-Responsive prodrug for real-time drug release monitoring and targeted cancer therapy

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Experimental section

Materials:

2-chlorotritylchloride resin (100–200 mesh, loading: 1.05 mmol g^{-1}), 2-(7-azo-benzotriazole)-*N,N,N',N'*-tetramethyluroniumhexafluorophosphate (HATU), o-benzotriazole-*N,N,N',N'*-tetramethyluroniumhexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), *N*-Fluorenyl-9-methoxycarbonyl (Fmoc) protected *L*-amino acids (FMOC-Arg(Pbf)-OH, FMOC-Gly-OH, FMOC-Asp(OtBu)-OH, FMOC-Ser(tBu)-OH, FMOC-Cys(Trt)-OH) and triisopropylsilane (TIS) were purchased from GL Biochem. Ltd. (Shanghai, China) and used as received. Diisopropylethylamine (DIEA) was acquired from GL Biochem. Ltd. (Shanghai, China) and used after distillation. Trifluoroacetic acid (TFA), phenol, piperidine, 1,2-ethanedithiol (EDT), *N,N'*-dimethylformamide (DMF), methanol, dichloromethane (DCM), tetrahydrofuran (THF), ethyl acetate, acetonitrile and anhydrous ether were obtained from Shanghai Chemical Co. (China). TFA, DMF and THF were used after distillation. 7-Hydroxycoumarinyl-4-acetic acid was purchased from Heowns Biochem. Technologies and used as received. Dialysis membrane (MWCO: 1000), 6-aminocaproic acid, maleic anhydride, 2,6-lutidine, *N*-methylmorpholine (NMM), isobutyl chloroformate, tert-butyl carbazate and 5(6)-Carboxyfluorescein were obtained from Aladdin Reagent Co. Ltd. (Shanghai, China), Doxorubicin hydrochloride (Dox·HCl) was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd (China). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (PBS), 3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium-bromide

(MTT), LysoSensor Green DND-189 and fetal bovine serum (FBS) were purchased from Invitrogen Corp. All other reagents were of analytical grade and used as received.

General characterizations:

Fluorescence spectra were recorded on a LS55 luminescence spectrometer (Perkin–Elmer). Electrospray ionization mass spectrometry was performed on LCQ Advantage, Finigan, USA. Fluorescence microscopy images were taken using a Carl Zeiss fluorescence microscopy, NOL-LSM 710. Absorbance spectra were recorded on a UV-Vis spectroscopy (Lambda Bio40).

Synthesis of precursor (peptide Cou-CGRGDS)

Cou-CGRGDS was synthesized manually by standard solid phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin and Fmoc-amino acids. After the resin was soaked in anhydrous DMF for half an hour, the first peptide residue was coupled to the resin using 0.5 equiv. (relative to the substitution degree of resin) of Fmoc-protected amino acid (FMOC-Ser(tBu)-OH) and 3 equiv. of DIEA in a DMF solution for 2 h. Unreacted sites of the resin were then capped by a 30 min incubation with a mixture of methanol, and DIEA in DMF (v/v/v=1:1:8), and then the resin was treated with 20% piperidine in DMF (v/v) for 15 min twice to remove the Fmoc protecting group in attached amino acid, and then the rest part of peptide residues were coupled in turn by reacting with 2 equiv. of Fmoc-protected amino acid, and 2.4 equiv. of

HBTU, 2.4 equiv. of HOBt, and 4 equiv. of DIEA for 2 h. After the coupling of amine acids, 7-hydroxycoumarinyl-4-acetic acid (1.5 equiv.) was conjugated to peptide segments using 1.35 equiv. of HATU, 1.8 equiv. of HOBt and 9 equiv. of DIEA in a DMF solution for 12 h. After the Cou-CGRGDS peptide was synthesized, the resin was washed with DMF, methanol and DCM for 4 times, respectively, and dried under vacuum overnight. Cleavage of the peptide from the resin was performed by stirring the dried resin with a mixture of TFA/phenol/thioanisole/H_2O/EDT (83: 6.3:4.3:4.3:2.1) for 2 h at room temperature. The filtration was further concentrated by rotary evaporation. The residue was obtained by precipitating the viscous solution in cold ether, collected by centrifugation, and then dried under vacuum. The solid obtained was conserved in a -20 ^{o}C freezer. The molecular weight of the obtained peptide (Cou-CGRGDS) was confirmed by electrospray ionization mass spectrometry (ESI-MS), calculated MS: 795.25, found $[M-H]^-$ = 794.2. The purity was confirmed by high performance liquid chromatography (HPLC).

Synthesis of (6-maleimidocaproyl)hydrazone of doxorubicin (Mal-hyd-Dox)

Mal-hyd-Dox was synthesized according to the literature reported by Firestone et al.[S1] (See Figure S1). The molecular weight of the obtained Mal-hyd-Dox was confirmed by electrospray ionization mass spectrometry (ESI-MS), calculated MS: 750.27, found $[M+H]^+$ =750.9. The purity was confirmed by high performance liquid chromatography (HPLC). ^{1}H NMR (CD_3SOCD3, 300 MHz): 7.93 (s, 1H), 7.81 (s,

1H), 7.68 (d, 1H), 7.00 (s, 2H), 5.50 (t, 1H), 4.97 (s, 1H), 4.42 (d, 1H), 4.25 (m, 1H), 4.00 (s, 3H), 3.52 (s, 2H), 3.29 (s, 1H), 3.23 (d, 2H), 2.75 (m, 1H), 2.4-2.25 (m, 3H), 2.20-1.77 (m, 3H), 1.70-1.03 (m, 12H).

Synthesis and purify of prodrug (Cou-C(Mal-hyd-Dox)-GRGDS)

Mal-hyd-Dox (1.1 equiv.) and Cou-CGRGDS (1 equiv.) were dissolved in DMF, and stirred at room temperature for 24 h. Then the product peptide solution was transferred to a dialysis membrane (MWCO: 1000), and purified by dialysis against DMF for 24 h. The DMF solution inside the dialysis bag was concentrated by rotary evaporation, and was precipitated in cold ether. The purified peptide was then collected by centrifugation, and dried under vacuum. The solid obtained was stored at a -20 ^{o}C freezer in the dark. The molecular weight of the peptide (Cou-C(Mal-hyd-Dox)-GRGDS) was confirmed by electrospray ionization mass spectrometry (ESI-MS), calculated MS: 1545.5, found $[M-H]^{-}$ = 1544.2. The purity was confirmed by high performance liquid chromatography (HPLC).

Fluorescence spectra and absorbance spectra of precursor and M-Prodrug (Cou-C(Mal-hyd-Dox)-GRGDS).

The precursor and M-Prodrug were dissolved in 10 mM sodium phosphate buffer (pH 7.4) and 10 mM acetate buffer solution (ABS, pH 5.0) at 20 μM, respectively.

Fluorescence emission spectra of these solutions were recorded just after dissolution (< 2 min, extremely less hydrazone bond breakage in this short time) at the excitation wavelength of 330 nm. Slit: 1.5 nm, 3 nm. Fluorescence excitation spectra of M-Prodrug solutions at pH 5.0 and 7.4 were also recorded just after dissolution (< 2 min) at the emission wavelength of 460 nm. Slit: 3 nm, 3 nm. Absorbance spectra were recorded just after dissolution, too (< 2 min).

Dox release monitored by fluorescence spectroscopy

M-Prodrug was dissolved in 10 mM sodium phosphate buffer (pH 7.4) and 10 mM acetate buffer solution (ABS, pH 5.0) at the concentration of 20 μM, respectively, and then 2 mL of these prodrug containing solution was transferred into the dialysis membrane (MWCO 1000). Subsequently, the dialysis membrane was immersed in 30 mL corresponding buffer solution and incubated at 37 ^{o}C. The incubation medium was analyzed by LS55 luminescence spectrometer (Perkin–Elmer) at given time intervals, slit: 10 nm, 10 nm.

Real-time Fluorescence Recovery

M-Prodrug was dissolved in 10 mM sodium phosphate buffer (pH 7.4) and 10 mM acetate buffer solution (ABS, pH 5.0) at the concentration of 20 μM, respectively. Fluorescence emission spectra of these solutions were recorded in different time

periods at the excitation wavelength of 330 nm, slit: 3 nm, 3 nm.

In Vitro Cytotoxicity

U87 cells and Hela cells were seeded into a 96 well plate (6000 cells/well) cultured in DMEM (200 μL) incubated for 24 h (37 ^{o}C, 5% CO_2), and then half of the culture medium was removed, DMEM (100 μL) containing a fixed concentration of M-Prodrug or free Dox (for comparison) were added in each well. The cells were co-incubated with prodrug or Dox solutions at 37 ^{o}C for 48 h. Then the drug contained DMEM media were replaced with 200 μL of fresh DMEM. MTT solution (20 μL, 5 mg/mL) was added to each well following and further incubated for another 4 h. Subsequently, the MTT media were removed and DMSO (200 μL) was added to each well. The optical density (OD) was measured at 570 nm with a microplate reader (BIO-RAD 550). The relatively cell viability was calculated as follows: Viability = $(OD_{sample}/OD_{control})\times100\%$, where OD_{sample} was obtained from the cells treated by M-Prodrug or free Dox and $OD_{control}$ was obtained from the cells without any treatments.

Confocal Laser Scanning Microscopy (CLSM)

U87 cells, Hela cells and COS7 cells were seeded in 35 mm Petri dishes and incubated in DMEM (1 mL) containing 10% FBS for 24 h. Thereafter, M-Prodrug dispersed in DMEM medium (1 mL) with 10% FBS at the Dox concentration of 10

μg/mL was added and the cells were further incubated at 37 ^{o}C for 1 h. After removing the medium and then washing with PBS (1 mL), the U87 cells, Hela cells and COS7 cells were fixed and observed by using Carl Zeiss fluorescence microscopy, NOL-LSM 710.

For real-time drug release monitoring by CLSM, U87 cells were seeded in 35 mm Petri dishes and incubated in DMEM (1 mL) containing 10% FBS for 24 h. Thereafter, M-Prodrug or a mixture of precursor and Dox dispersed in DMEM medium (1 mL) with 10% FBS at the Dox concentration of 5 μg/mL was added and the cells were further incubated at 37 ^{o}C for 0.5 h. , the drug contained DMEM media were replaced with 1 mL of fresh DMEM containing 10% FBS further incubated for another 0 h, 4 h and 12 h respectively, to allow the sufficient intracellular drug release of M-Prodrug. After removing the medium and washing twice with PBS, U87 cells were stained with 1 μM LysoSensor Green DND-189 for 15 minutes in the dark, and then removing the medium and washing thrice with PBS. The cells were then transferred into serum free media, and the cells images were taken by Carl Zeiss fluorescence microscopy, NOL-LSM 710.

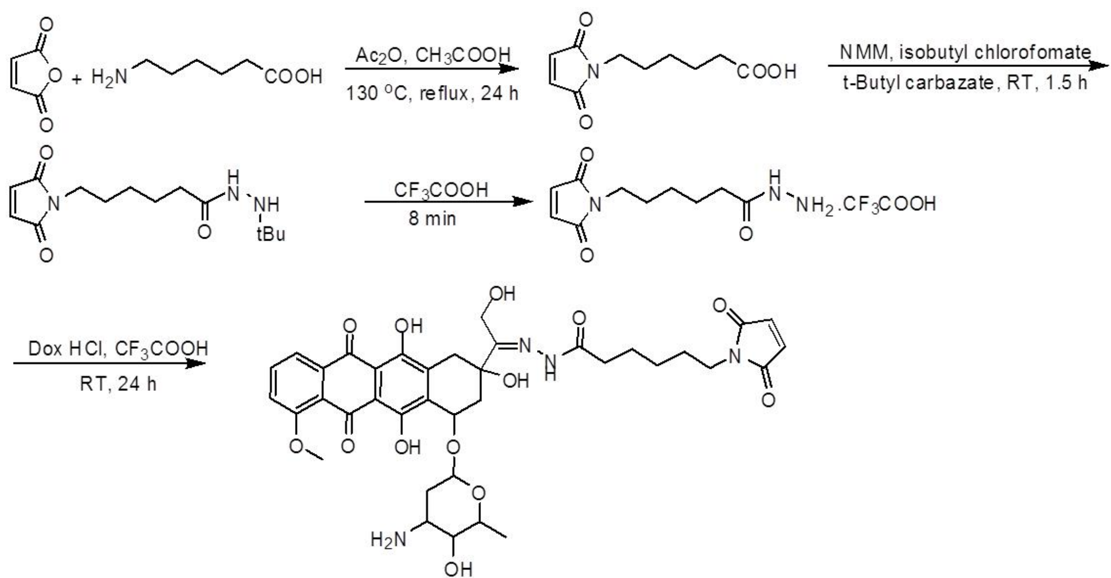

Fig. S1 Synthesis of (6-maleimidocaproyl)hydrazone of doxorubicin (Mal-hyd-Dox).

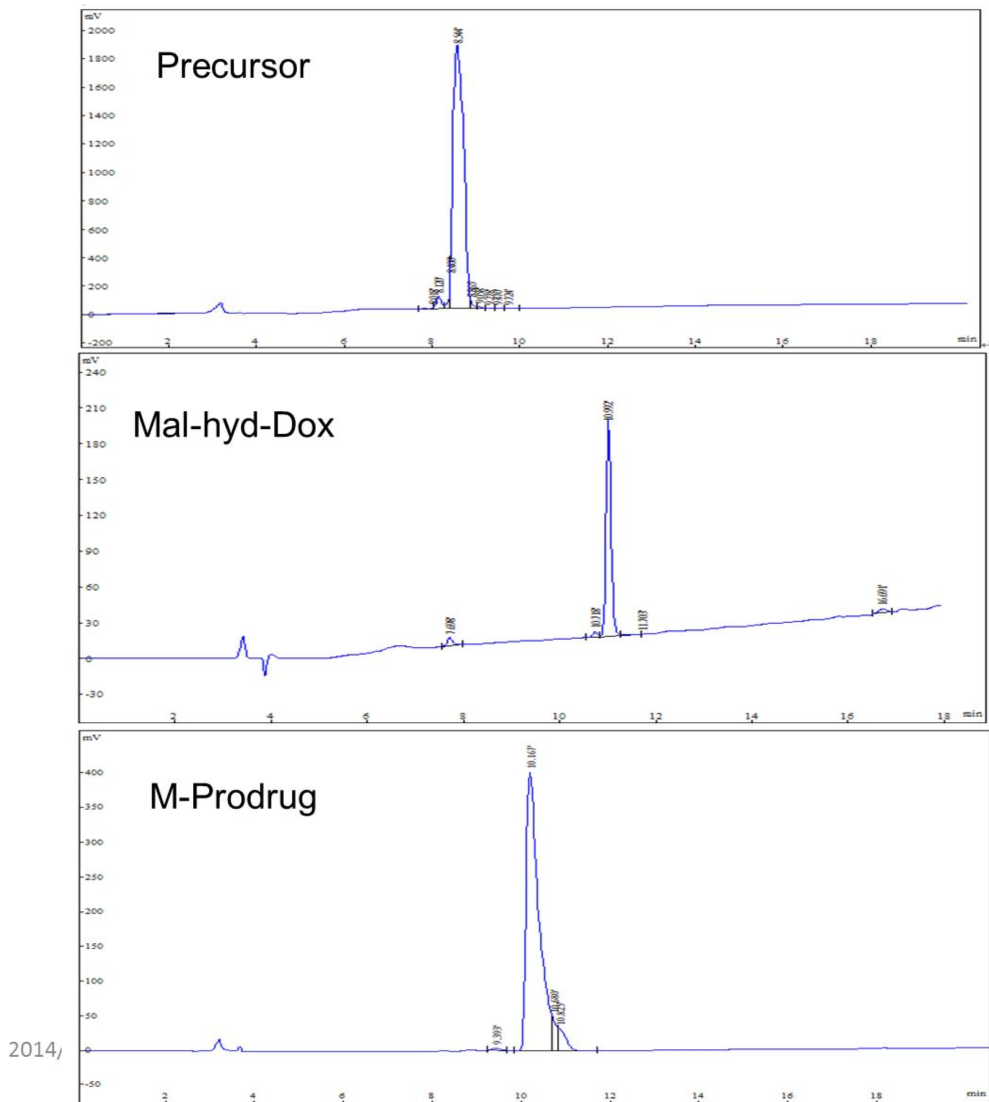

Fig. S2 HPLC analyses of Precursor (peptide Cou-CGRGDS), Mal-hyd-Dox and M-Prodrug.

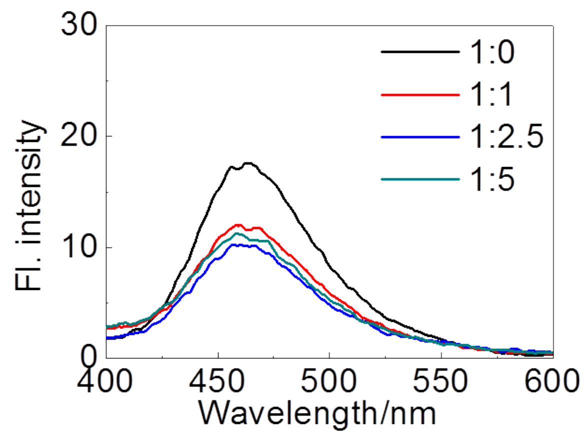

Fig. S3 Fluorescent intensity of Precursor (peptide Cou-CGRGDS) incubated with increasing concentration of Mal-hyd-Dox.

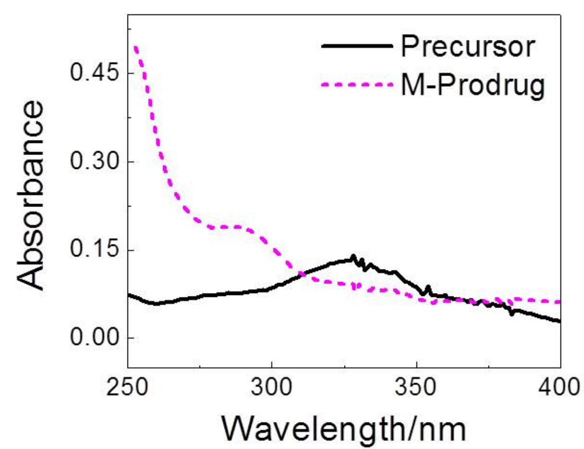

Fig. S4 Absorption spectra of the precursor and M-Prodrug just after dissolution (< 2 min) in 10 mM sodium phosphate buffer (pH 7.4) at the concentration of 20 μM.

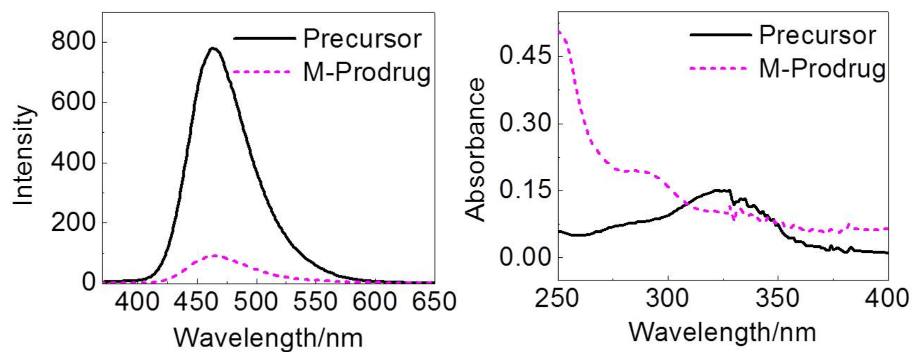

Fig. S5 Fluorescence (a) and absorption (b) spectra of the precursor and M-Prodrug just after dissolution (< 2 min) in 10 mM ABS (pH 5.0) at the concentration of 20 μM.

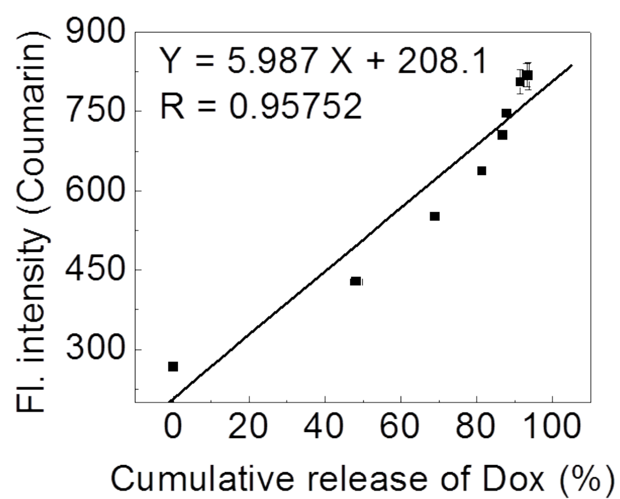

Fig. S6 Changes of fluorescent intensity of coumarin to cumulating release of Dox.

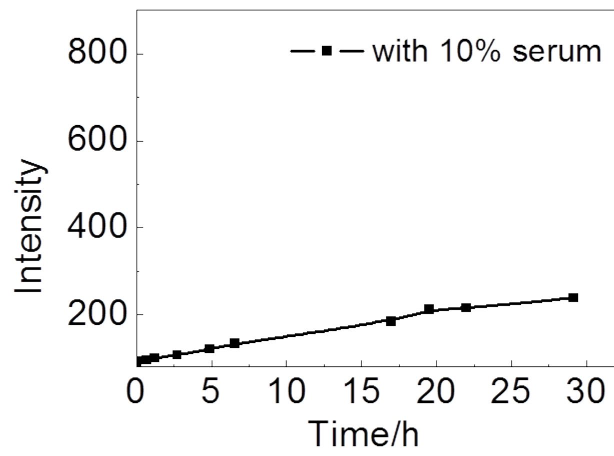

Fig. S7 Fluorescent intensity of M-Prodrug (10 μM) dissolved in 10 mM phosphate buffer solution (pH 7.4) containing 10% serum at different time periods.

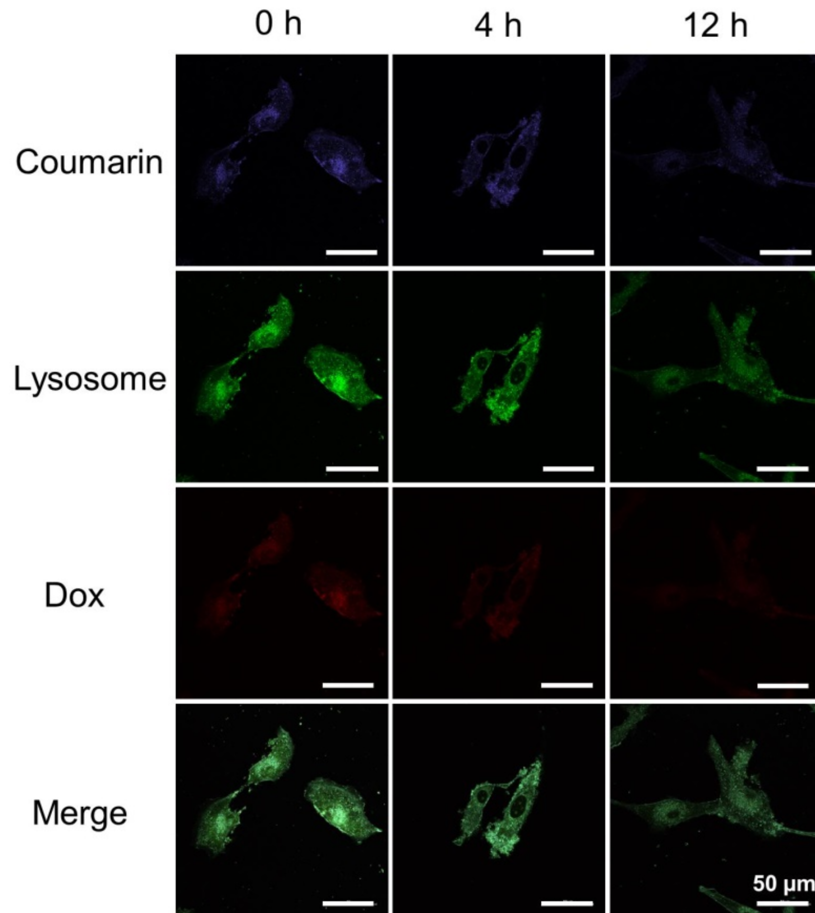

Fig. S8 Confocal microscopy images of U87 cells treated with a mixture of precursor and Mal-hyd-Dox at the Dox concentration of 5 μg/mL. Scale bar, 50 μm. Images were obtained using two-photon excitation wavelength of 750 nm. The lysosomes were stained with LysoSensor Green DND-189 (green fluorescence).

Reference

S1. D. Willner, P. A. Trail, S. J. Hofstead, H. D. King, S. J. Lasch, G. R. Braslawsky, R. S. Greenfield, T. Kaneko, and R. A. Firestone, *Bioconjugate Chem.,* 1993, **4**, 521.