Electronic Supporting Information

Using a peptide segment to covalently conjugate Doxorubicin and Taxol for the study of drug combination effect

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EXPERIMENTAL SECTION

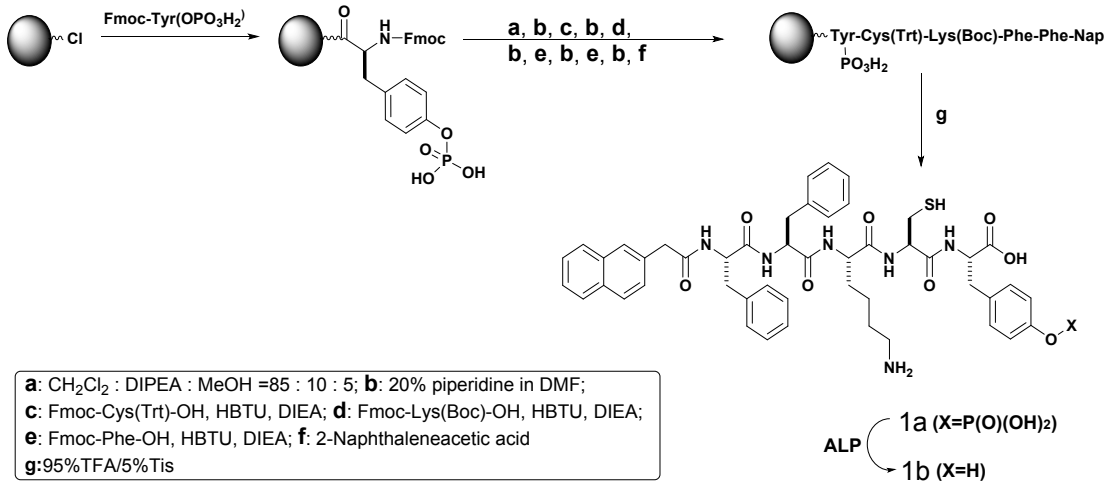
1. Materials and methods

All chemical reagents and solvents were obtained from commercial sources. The N-Fmoc-protected amino acid and 2-chlorotrityl chloride resin were acquired from GL Biochem Ltd (Shanghai, China). Taxol and Doxorubicin were purchased from DaLian Meilun Biotech Co.Ltd (Dalian, China). 1-Hydroxybenzotriazole (HOBT), 0-benzotriazole- N,N,N',N' -tetramethyl-uroniumhexafluoro-phosphate (TBTU), N,N-diisopropylethylamine (DIPEA), 4-dimethylamino-pyridine (DMAP) and trifluoroacetic acid (TFA), Triisopropylsilane (TIS), N-hydroxysuccinimide (NHS) were obtained from Aladdin Reagent Corporation (Shanghai, China). Alkali phosphatase (3.8U/mg) were purchased from J&K Scientific chemical Ltd (Beijing, China). The human breast cancer stem cells MCF-7 cells were gained from American Type Culture Collection (ATCC, America). MAD-MB-231 cells were from Cell research institute library of Shanghai. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Biosharp Company.

2. Design and synthesis

2.1 The synthesis of peptide (1)

The synthesis of **1a** was quite simple and straightforward via Fmoc protected amino acid based solid phase peptide synthesis (SPPS).[1, 2] Firstly, 0.2mmol 2-chlorotrityl chloride resin (1.0~1.2 mmol/g) was swelled in dry DCM for 20 minutes and then washed by dry DCM three times. We prepare Fmoc-phosphotyrosine (2 equiv.) by protecting the α-amine group of phosphotyrosine using Fmoc-Osu and DIPEA（2 equiv.) to react for 2 hours. Then washed with DCM three times, the unreacted sites in resin were blocked with DCM/MeOH/DIPEA solution (80: 15: 5) for 20 minutes and washed by DMF five times. Then the 20% piperidine (in DMF) was added for 30 min to remove the protecting group, followed by washing the resin with DMF five times. After loading Fmoc-phosphotyrosine on the 2-chlorotrityl chloride resin, we carried out the elongation steps according to the typical SPPS method to get the desired peptide sequence on the resin. The molecule **1a** was cleaved off by reagent TFA/TIS (95:5), concentrated the filter solution on rotary evaporator and poured into ice-cold ether. The resulting precipitate was centrifuged for 10 min at 13,000 rpm. Afterward, the supernatant was removed and lyophilized in double-distilled water to obtain white-light yellow precipitate, purified by HPLC(Shimadzu, Japan) and lyophilized to yield white power .^{1}H NMR (400MHZ, DMSO-d_6): δ 8.44-8.05 (m, 5H), δ 7.85 (d, 1H), δ 7.77 (d, 1H), δ7.73 (d, 1H), δ 7.57 (s,1H), δ 7.47 (m, 2H), δ 7.30-7.01 (m, 10H), δ 4.63-4.22 (m, 5H), δ 3.52 (q, 2H), δ 3.12-2.56 (m, 10H), δ 1.70-1.07 (m, 11H); TOF-MS: calcd. m/z 955.0, obsvd. $[M-2H]^{2-}$ 953.5, $[M-H]^-$ 954.5.



Scheme S1 The synthetic route of **1a** via SPPS based on Fmoc-strategy.

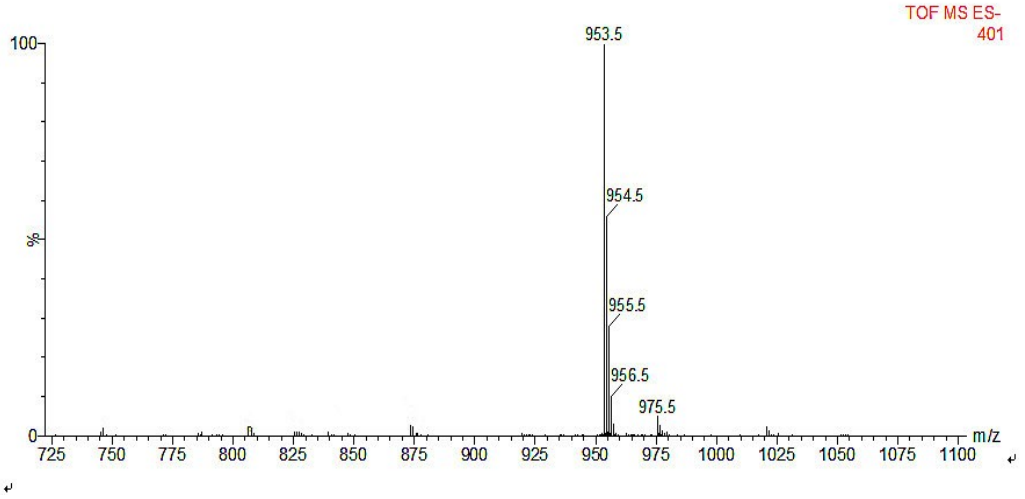


Figure S1 MS spectrum of peptide Nap-FFKCYp.

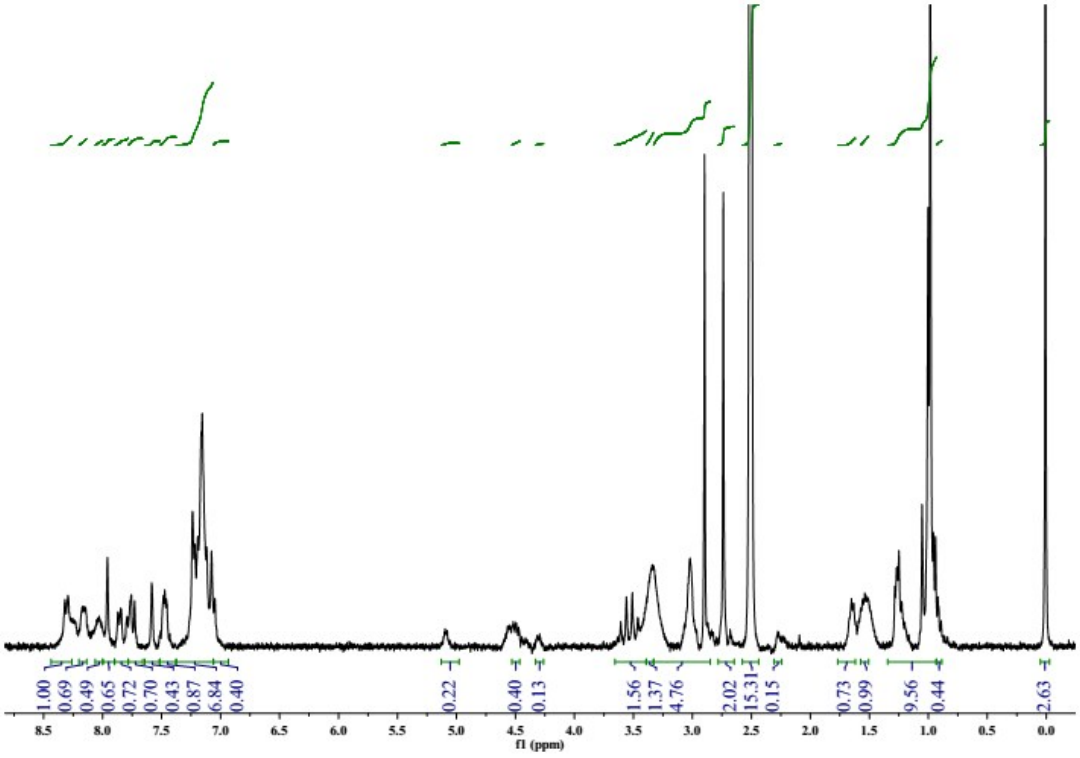


Figure S2 ^{1}H NMR spectrum of peptide Nap-FFKCYp.

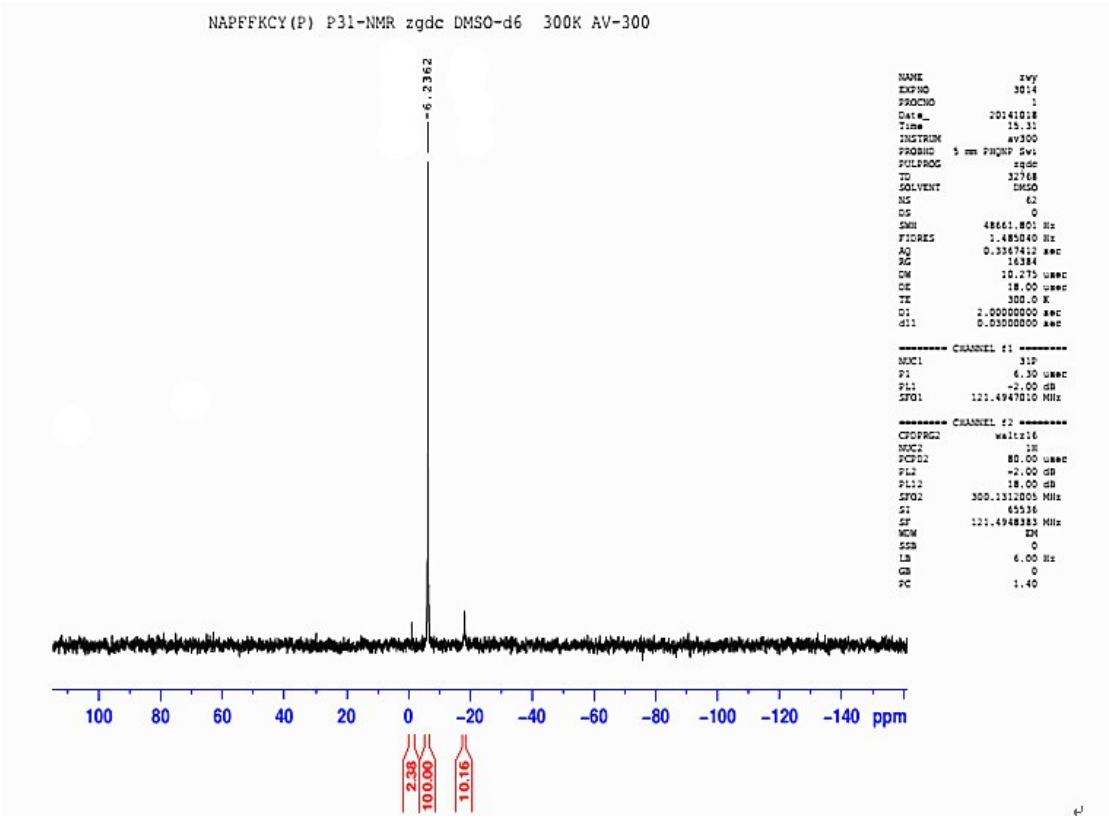


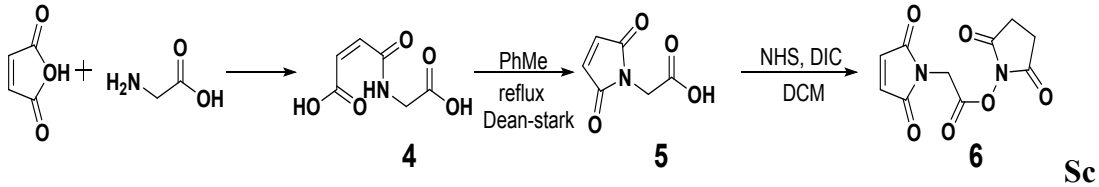
Figure S3 ^{31}P NMR spectrum of peptide Nap-FFKCYp.

2.2 Doxorubicin-maleimide derivative (2)

To synthesize the Doxorubicin-maleimide derivative, first we tried to react Dox with N-(methoxycarbonyl) maleimide. Due to the structure of Dox collapses quickly at strong basic condition, we failed to find a suitable reaction to make this reaction work. Alternatively, the structure of Dox is altered at C-13 via a Schiff base or 3'-NH_2 via an amide bond.[3] Due to the instability of Schiff base, we preferred the amide strategy to derive the maleimide group from the 3'-NH_2. Also the reported maleimide containing reactant was hard to prepare, we chose to connect a simply linker (glycine) to afford MA-Gly-NHS (**6**).

An intermediate, 2-(2,5-dioxo-2H-pyrrol-1(5H)-yl) acetic acid (**4**), was first synthesized based on the method described by Kevin G. Rice group with minor modification.[4] A mixture of equivalents amount of maleic anhydride (66.6mmol) and glycine(66.6mmol) were refluxed in 80 ml of acetic acid for 3 hours, collecting the resulting white precipitate by filtration， washed by addition of 20 ml of cold water and dried, (TLC 2:1:1:1 isopropyl alcohol/acetic acid/ethyl acetate/water, Rf =0.5) The

mixture was recrystallized from ethanol to afford white solid. According to ^{1}H NMR spectrum, a pair of doublet peaks at δ=6.41 and δ=6.32 indicated that there were two different olefinic protons. ^{1}H NMR (400 MHz, DMSO-d6): δ 13.49 (d, 2H), δ 9.22 (s, 1H), 6.41 (d, 1H, J=12Hz), 6.32 (d, 1H, J=12Hz), 3.90 (d, 2H, J= 8Hz).



Sc

heme S2 The synthetic route of MA-Gly-NHS (**6**).

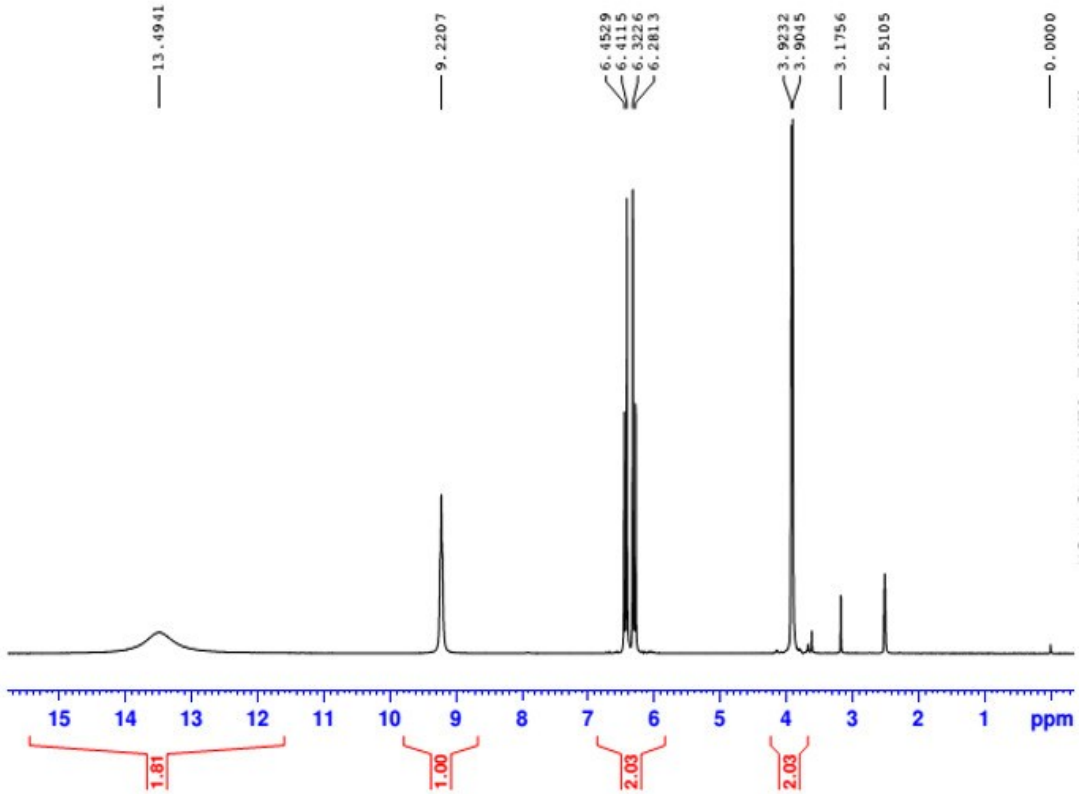


Figure S4 ^{1}H NMR spectrum of GMA **(4)**.

We continued the reaction according to Daniel' s method.[5] **4** (2.91g,16.8mmol) was suspended in dry toluene (500ml) with the addition of Et_3N (3.55 g,4.86 ml,35.1mmol). This mixed solution was reflowed with strong agitating for 1 hour with concomitant removal of formed H_2O via a Dean-Stark apparatus. The toluene was removed by evaporation. The solid was acidified with HCl to pH=2 and extracted with EtOAc, finally dried with $MgSO_4$. The organic phase was dried in vacuum to give final product

compound **5**. ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.16 (s, -COOH), 7.12 (s, 2H), 4.13(s, 2H). That is different from ^{1}H NMR of **4.**

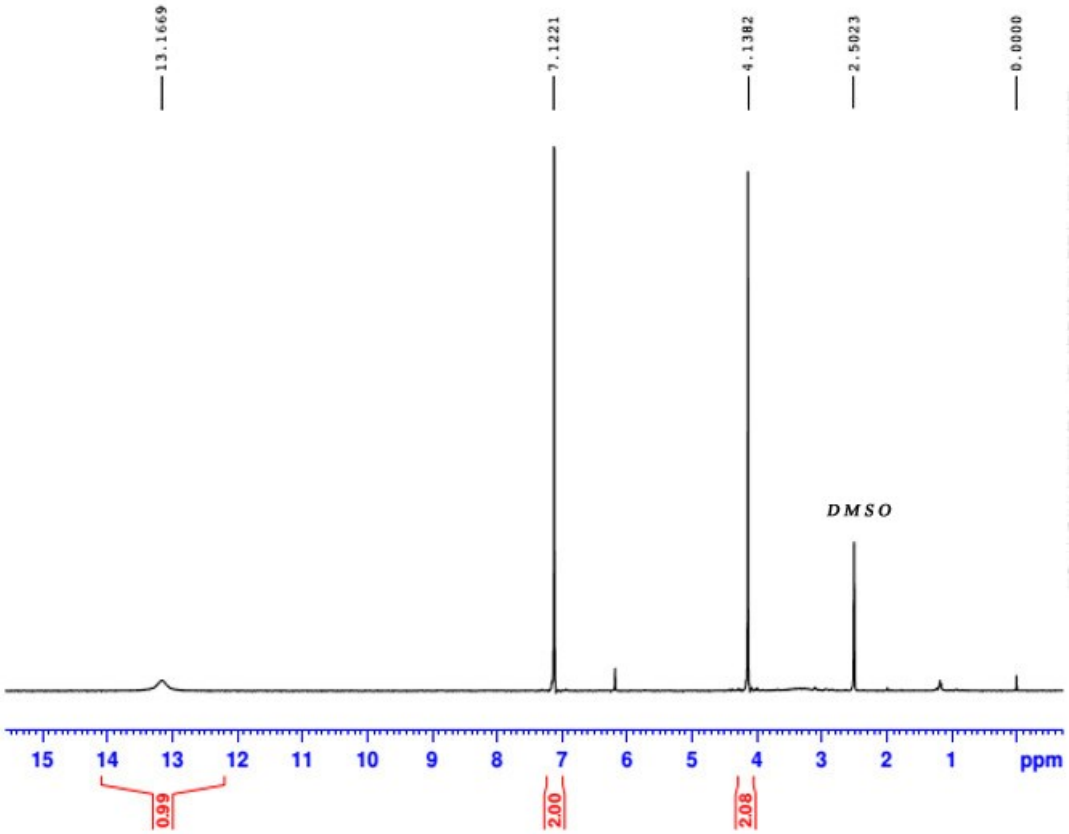


Figure S5 ^{1}H NMR spectrum of GMI **(5)**.

(2, 5 -Dioxo-2, 5-dihydro-pyrrol-1-yl)- acetic acid 2,5 -dioxo-pyrrolidin-1-yl ester (Gly-MA-NHS, **6**) was synthesized by a previously reported method with slighter modification.[6, 7] Compound **5** (2mmol) and NHS (2mmol) were suspended in 10 mL of THF (10ml), then followed by addition of DIC (2 mmol) in THF (10ml) while stirring at room temperature. The reaction completed in three hours with TLC monitoring. After filtrating to remove the precipitate, the acquired filtrate was concentrated under vacuum, washed by EtOH twice and re-suspended in 2-propanol (10ml) while stirring for 1 hour, and then filtered to collect the precipitate. The precipitate was washed by 2-propanol and dried to yield a white solid compound **6**. ^{1}H NMR (400MH$_Z$, DMSO-d_6): δ 7.19 (s, 2H), 4.73 (s, 2H), 2.81 (s, 4H). MS also confirmed the formation of **6**. TOF-MS: calcd.m/z 252.2, obsvd. $[M+Na]^+$ 275.0.

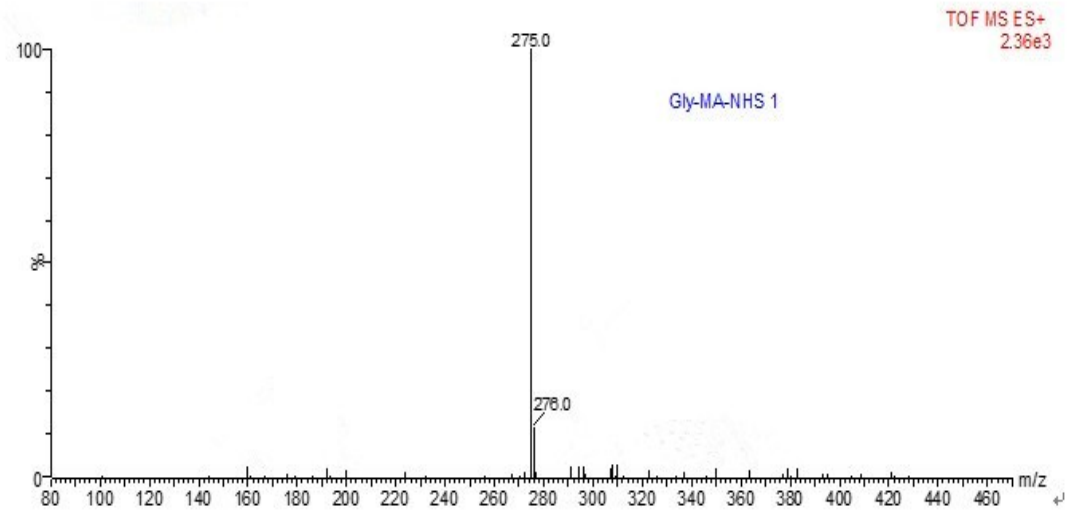


Figure S6 MS spectrum of MA-Gly-NHS **(6)**.

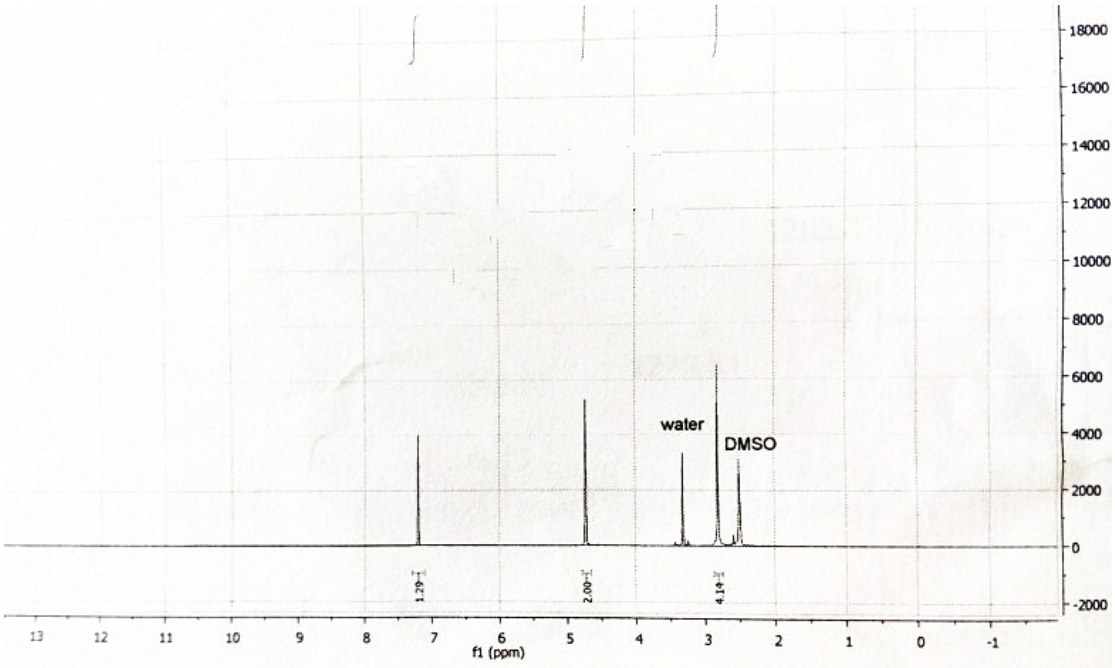
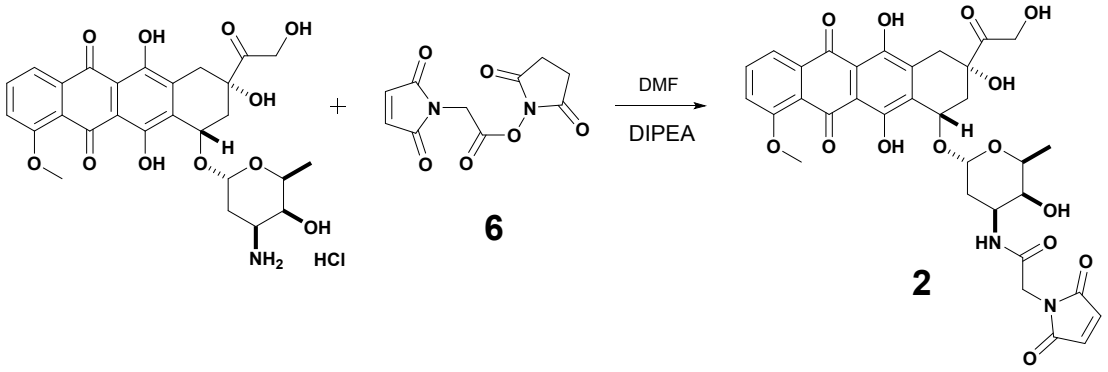


Figure S7 ^{1}H NMR spectrum of MA-Gly-NHS **(6)**.

Doxo-Gly-MA (**2**) was synthesized via a simple ester-amide exchange reaction.[8] To a 25 mL round-bottom flask anhydrous DMF (5.3 ml), Doxorubicin HCl salt (50 mg, 86.2µmol), and diisopropylethylamine (75µl, 430.5µmol) was sequentially added. The reaction flask was wrapped with aluminum foil and the mixture was stirred at room temperature under nitrogen for 10 min to get all solid dissolved to yield a dark red but transparent solution. This solution was then transferred to an addition funnel and added

dropwisely into the solution of **6** (21.7mg, 86.1μmol) in anhydrous DMF (1.3 ml) containing diisopropylethylamine completed in 2 hours at room temperature according to TLC monitoring (DCM/ MeOH/AcOH, 6/2/2, v/v/v). The solvent was then removed under vacuum at 30-35 ℃. The oily reddish residue was then mixed with anhydrous ether (50 ml) to achieve a turbid solution. The suspension was applied to sonication for the complete precipitation. The resulting precipitation was collected by suction filtration and washed by cold ether. The product was further purified by the following procedure three times: the crude product was dissolved in DCM with assistance of an ultrasonic bath and filter to get rid of any insoluble solid (un-reacted dox is not soluble in dichloromethane, so it can removed by suction filtration). Then the reddish solution was concentrated to 1/10 of the original volume. Add the anhydrous ether dropwisely to precipitate and get out the product. The solid was then collected and dried. ^{1}H NMR (400 MHz, DMSO-d_6): δ 14.00 (s, 1H), 13.24 (s, 1H), 7.90 (m,2H), 7.65 (m, 2H), 7.02 (s, 2H), 5.43 (s, 1H), 5.23 (d, 1H, J= 2.5 Hz), 4.98-4.86 (m, 2H), 4.72 (d, 1H, J= 4.5 Hz), 4.56 (d, 2H, J= 5.4 Hz), 4.17 (m, 2H), 3.98 (s, 5H), 3.36 (m, 1H), 2.22-2.07 (m, 2H), 1.91-1.79 (m, 1H), 1.47-1.39 (m, 1H), 1.16-1.07 (m, 3H); TOF-MS: calcd. m/z 680.2, obsvd. [M-H]$^-$ 679.3.



Scheme S3 The synthesis of Dox-Gly-MA (**2**).

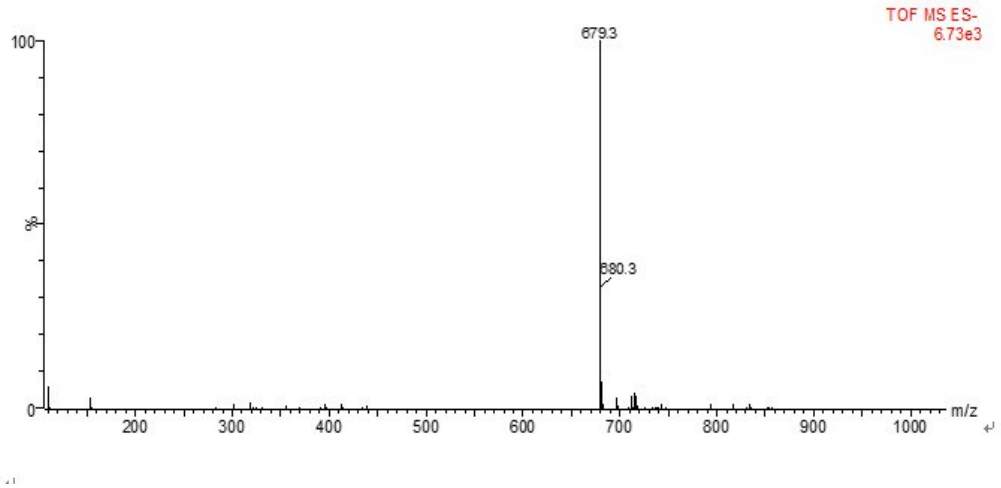


Figure S8 MS spectrum of Dox-Gly-MA (2).

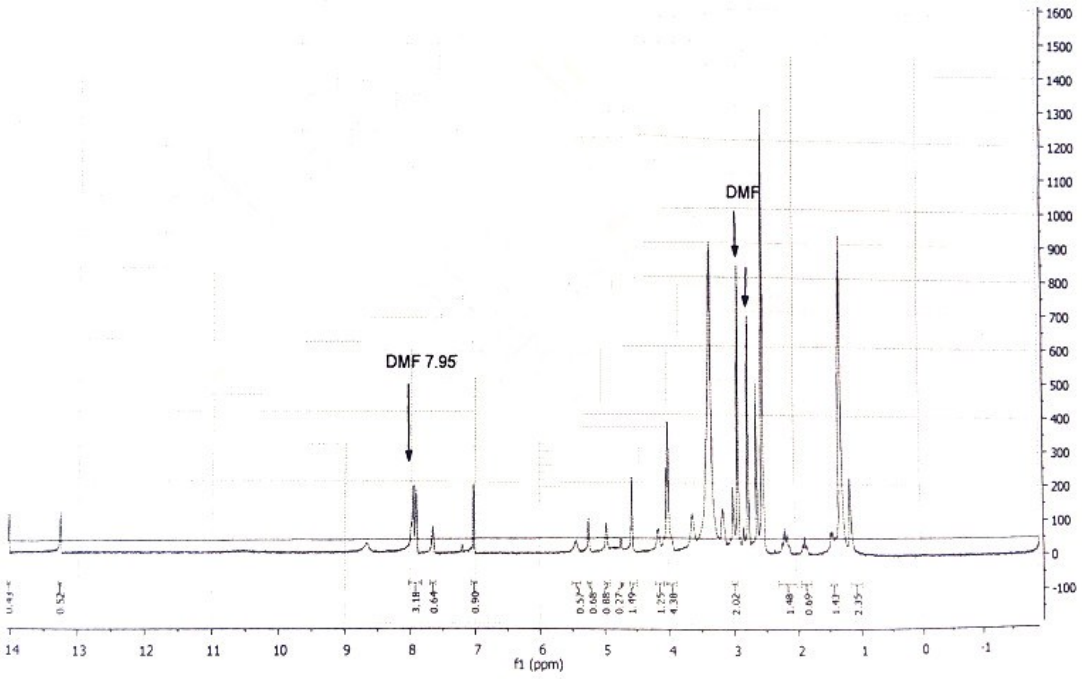
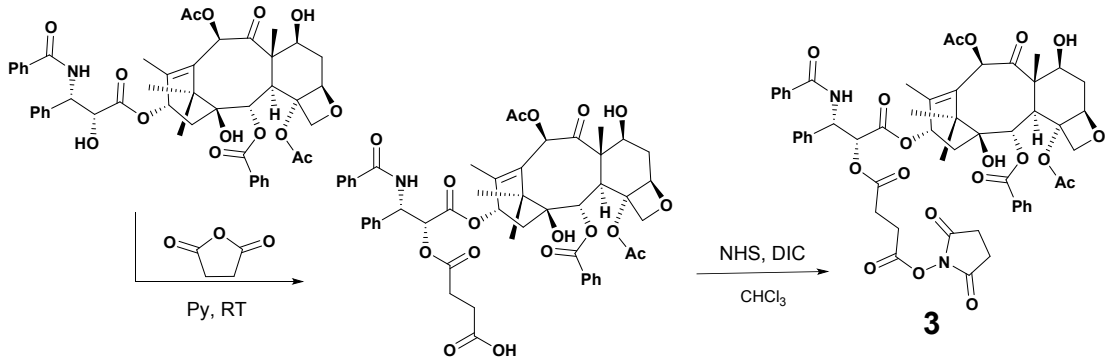


Figure S9 ^{1}H NMR spectrum of Dox-Gly-MA (2).

2.3 The synthesis of Taxol-Succi-NHS active ester (3)

The Taxol-Succi-NHS**(3)**, which was synthesized in advance according to previous study,[9, 10] was connected to the ε-NH_2 of the lysine on compound **7** via a ester-amide exchange reaction to afford the product compound **3** . Taxol（170.6mg, 0.2mmol）and siccinic anhydride (70mg, 0.7mmol) was added to 5ml of dry pyridine and stirred for 3 hours in the presence of 4-dimethylamino-pyridine (41mg, 0.33mmol) which was previously dried under vacuum for 2 hours. The product was purified by extraction as following: after the completion of reacting, 20ml of dry dichloromethane (DCM) was

added into the mixture, the organic phase was washed by 1M HCl (20ml) and water (20ml) for three tines. After that, water phase was washed by 10ml DCM for three times. Combine the organic phase and washed by 10ml brine for three times and dried over Na_2SO_4. The filtrate was concentrated on rotary evaporator to get compound **3**. Compound **3** (190.6mg, 0.2mmol) and N-hydroxysuccinimide (23.0mg,0.2mmol) was mixed in 10ml $CHCl_3$, then N,N-Dicylcohexylcarbodiimide (41.2mg, 0.2mmol) was added into the solution and allowed to react for 4 hours at room temperature. Finally, the 2-NHS-siccinyl-Taxol **(3)** was purified by chromatography (TLC, Rf =0.68, DCM /MeOH, 10:1). ^{1}H NMR (300 MHz, CDCl3) δ 8.66 (s, 1H), 8.15 (d, *J* = 7.4Hz, 2H), 7.85-7.67 (m, 1H), 7.66-7.21 (m, 11H), 7.20-7.01 (m, 1H), 6.34-6.11 (m, 2H),6.05-5.91 (m, 1H), 5.68 (d, *J* = 7.1 Hz, 1H), 5.54 (t, *J* = 6.0 Hz, 1H), 4.98 (d, *J* = 9.1 Hz, 1H),4.52-4.38 (m, 1H), 4.31 (d, *J* = 8.3 Hz, 1H), 4.21 (d, *J* = 8.3 Hz, 1H), 3.94-3.75 (m, 6H), 3.02-2.61 (m, 6H), 2.58-2.00 (m, 6H), 1.99-1.56 (m, 7H), 1.20 (dd, *J* = 29.8, 7.5 Hz, 35H). TOF-MS: calcd. m/z 1051.1 obsvd. $[M-2H]^{2-}$ 1049.5, $[M-H]^-$ 1050.5.



Scheme S4 The synthetic route of Taxol-Succi-NHS **(3)**.

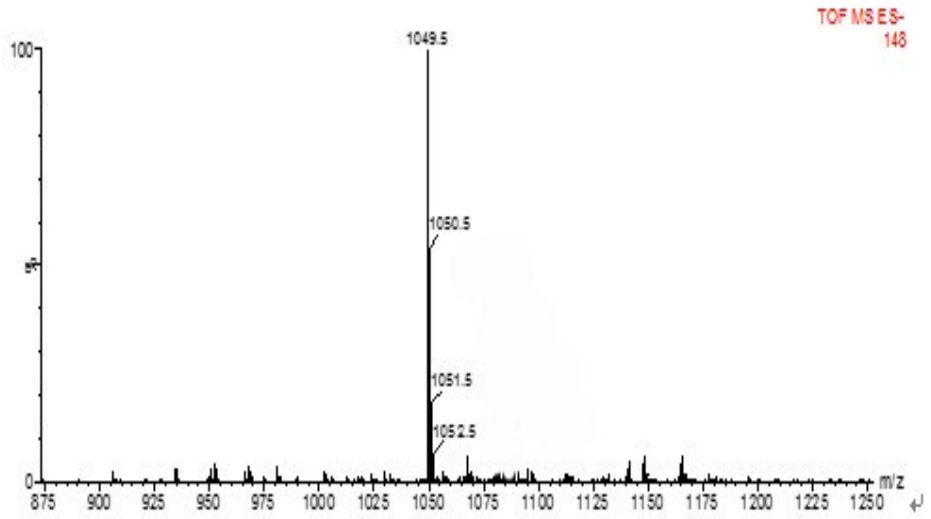


Figure S10 MS spectrum of Taxol-Succi-NHS **(3)**.

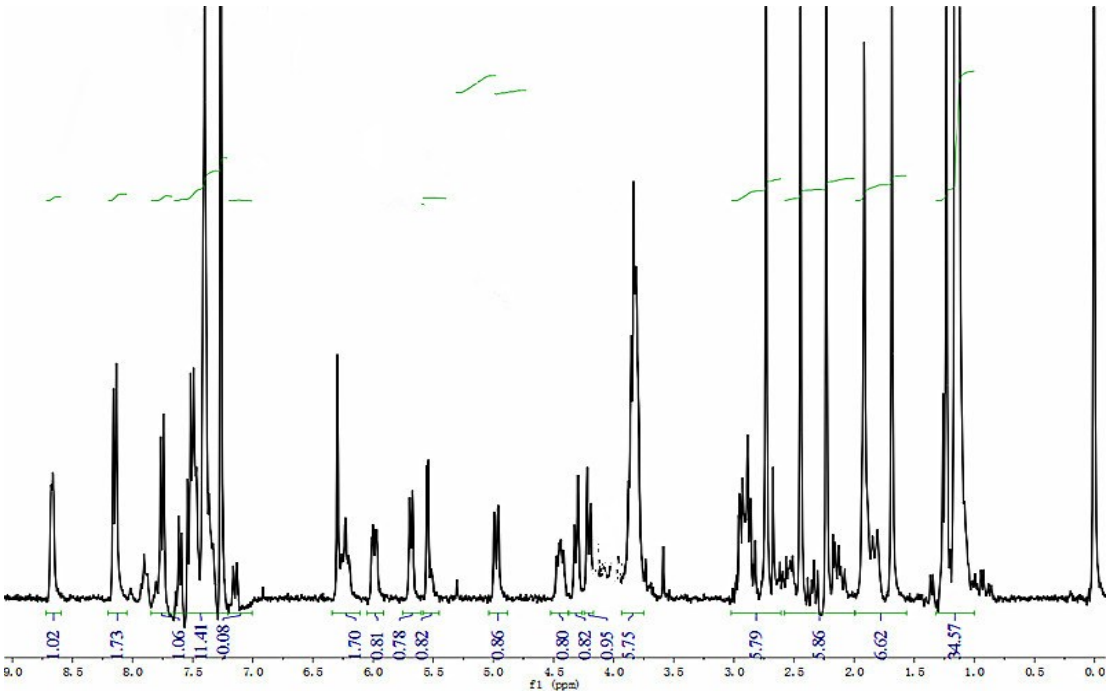
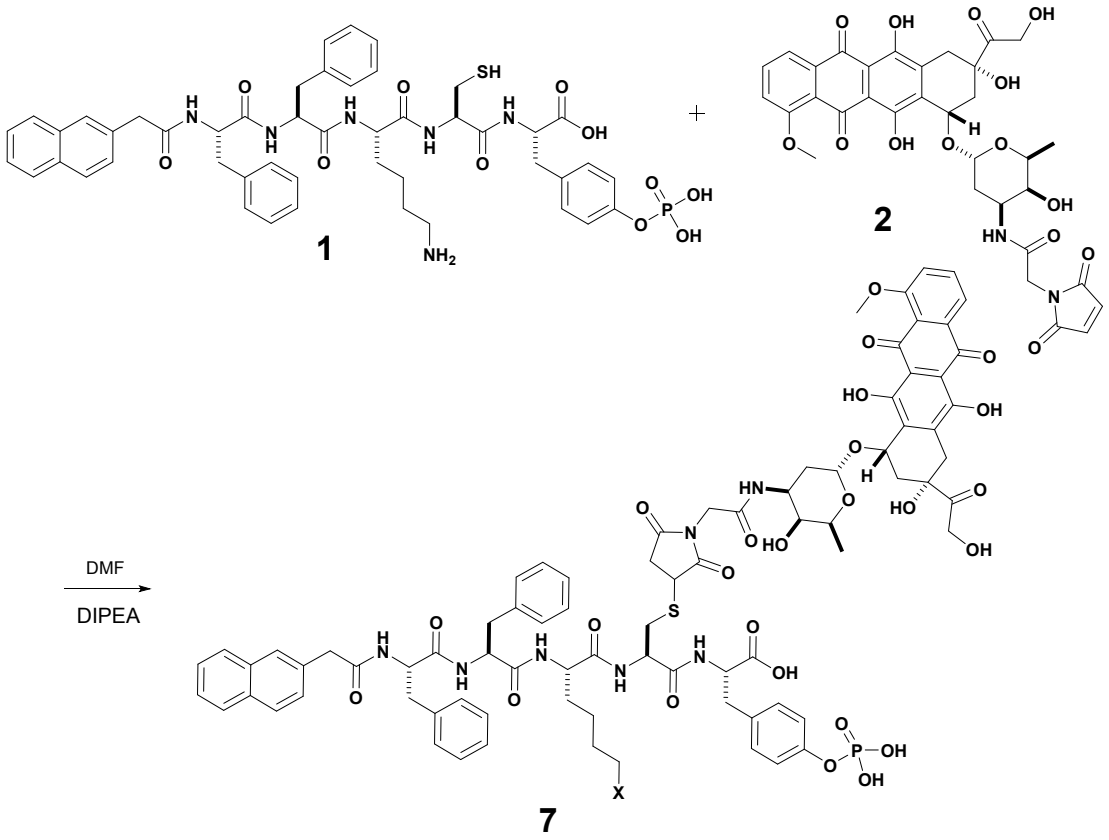


Figure S11 ^{1}H NMR spectrum of Taxol-succi-NHS **(3)**.

2.4 Preparation of NapFFKC (dox) Yp (7)

7 was synthesized by connect **1a** and **2** via a Michael addition: **2** (20mg) was dissolved in 1.5mL of PBS) with the addition of TCEP (4.6mg). During the mixing step, the PH of the solution was maintained at 7 with the addition of 1M NaOH to avoid the gel formation. The mixed solution was stirred for two hours at room temperature to allow

the reaction completed. Compound **7** was then separated by HPLC in a reverse phase C-18 column with the elution at 5.0 ml/min using water-CH_3CN with 0.1% TFA (from 7:3 to 2:8 over 30min) and detection at 276 nm. Fraction of right peptide were combined and lyophilized to provide a red powder. ^1H NMR (400 MHz, DMSO-d_6): δ 14.00 (s, 1H), 13.24 (s, 1H), 8.44-8.05 (m, 5H), 7.90 (m,2H), 7.85 (d, 1H),7.77 (d, 1H), 7.73 (d, 1H), 7.65 (m, 2H), 7.57 (s, 1H), 7.47 (m, 2H), 7.30-7.01 (m, 15H), 5.43 (s, 1H), 5.23 (d, 1H, J= 2.5 Hz), 4.98-4.86 (m, 2H), 4.72 (d, 1H, J= 4.5 Hz), 4.56 (d, 2H, J= 5.4 Hz), 4.63-4.22 (m, 5H), 4.17 (m, 2H), 3.98 (m, 6H), 3.52 (q, 2H), 3.36 (m, 1H), 3.12-2.56 (m, 12H), 2.22-2.07 (m, 2H), 1.91-1.79 (m, 1H), 1.70-1.07 (m, 10H); ESI MS: calcd. m/z 1634.2 obsvd. $[M+2H]^{2+}$ 1636.8.



Scheme S5 The synthetic route of NapFFKC (dox) Yp (**7**).

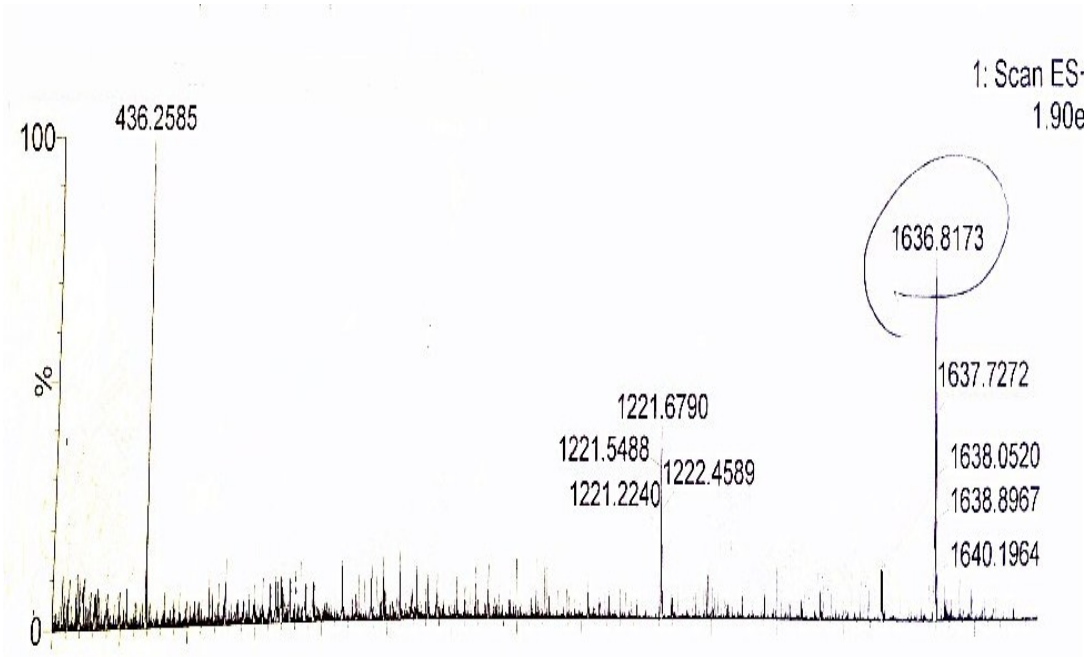


Figure S12 MS spectrum of NapFFKC (dox) Yp (**7**).

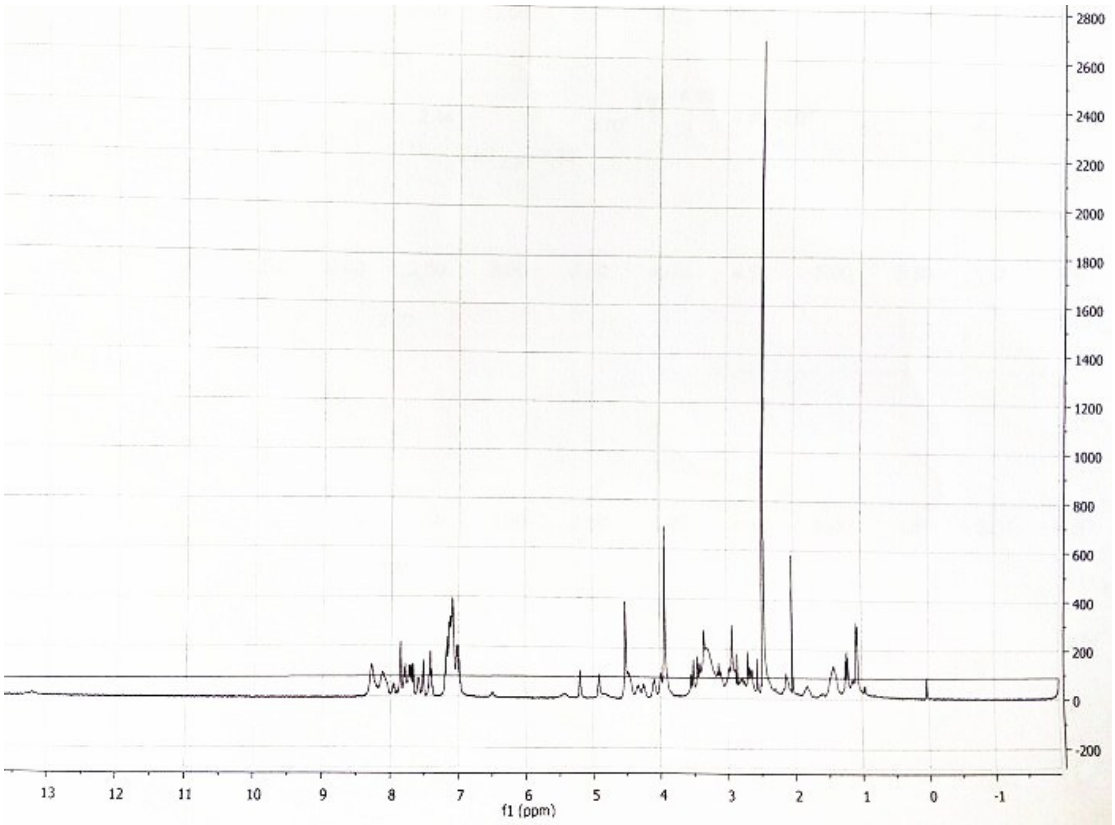
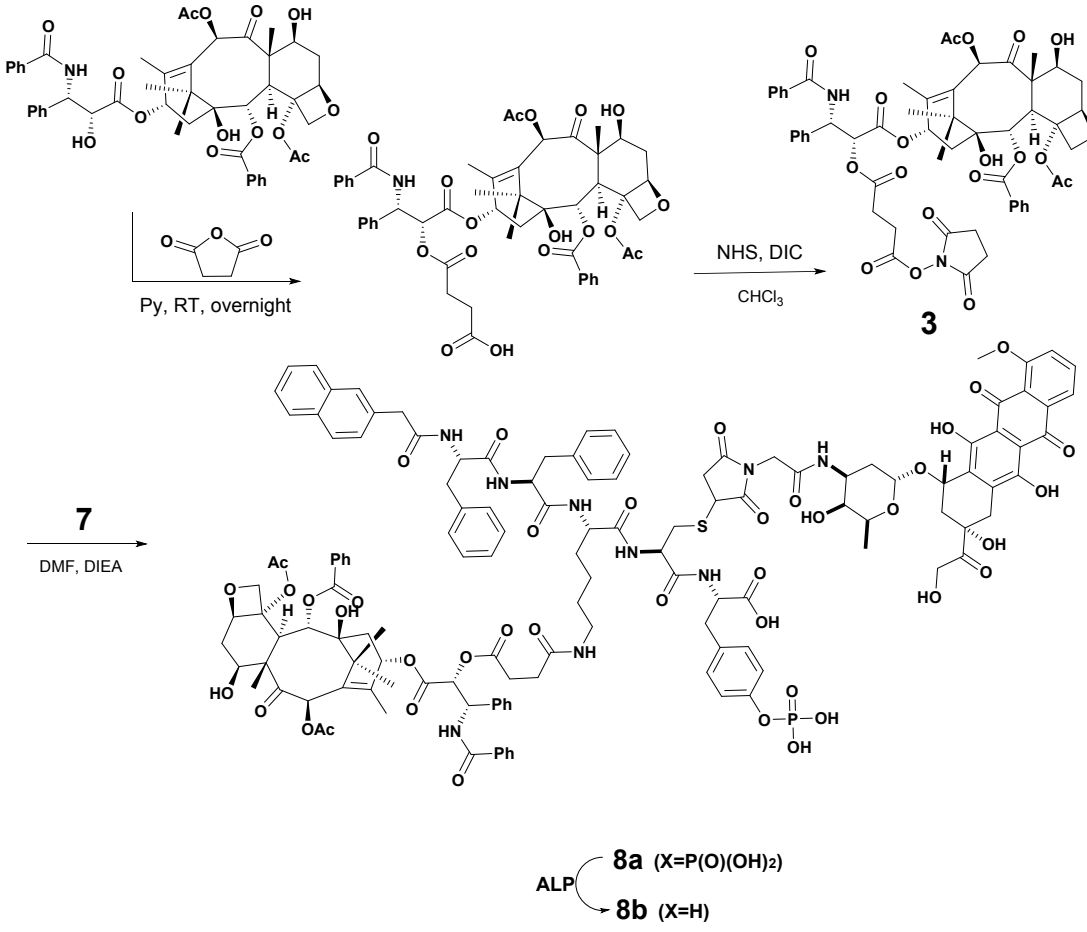


Figure S13 ^{1}H NMR spectrum of NapFFKC (dox) Yp (**7**).

2.5 The synthesis of NapFFK (T) C (D) Yp (8)

Compound **3** (12.8mg) was dissolved in DMF with an addition of DIPEA (4mg). Compound **7** (9.6mg) was dissolved in DMF with another 12 mg of DIPEA. The solution of **3** and **7** were mixed in a flask wrapped with aluminum foil. According to

TLC monitoring (DCM/MeOH, 8/2, v/v), the reaction completed over night at room temperature. The reaction mixture was separated by HPLC with the method same as that for the purification of compound **7** to afford compound **8**. ^{1}H NMR (400 MHz, DMSO-d6): δ 14.00 (s, 1H), 13.24 (s, 1H), 9.19 (d, 1H), 8.44-8.05 (m, 6H), 7.90-7.85 (m, 7H), 7.77-7.01 (m, 34H), 6.28 (s, 1H), 5.82 (t, 1H), 5.54 (t, 2H), 5.43 (s, 1H), 5.33 (d, 1H), 5.23 (d, 1H, J= 2.5 Hz), 4.98-4.86 (m, 4H), 4.72-4.55(m, 4H), 4.56 (d, 2H, J= 5.4 Hz), 4.63-4.22 (m, 5H), 4.17-3.98 (m, 12H), 3.52 (q, 2H), 3.36 (m, 1H), 3.12-2.56 (m, 15H), 2.48-2.26 (m, 3H), 2.23 (s, 3H), 2.22–2.07 (m, 5H), 1.91-1.79 (m, 2H), 1.76 (s, 3H), 1.49 (s, 3H), 1.70-1.07 (m, 15H), 1.00 (d, 3H); ESI MS: calcd. m/z 2569.6 obsvd. $[M+H]^{+}$ 2570.5 and [M+Na-H] 2592.4.



Scheme S6 The synthetic route of NapFFK(taxol)C(dox)Yp **(8)**.

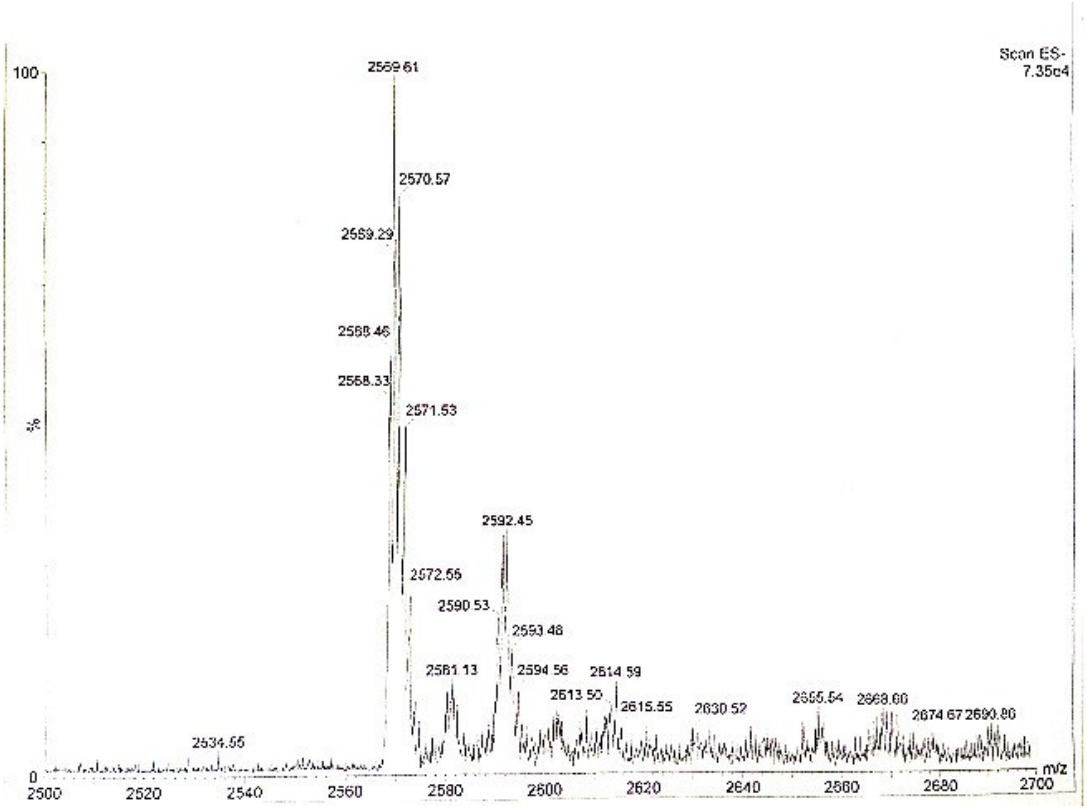


Figure S14 MS spectrum of Nap FFK (taxol) C (dox) Yp **(8)**.

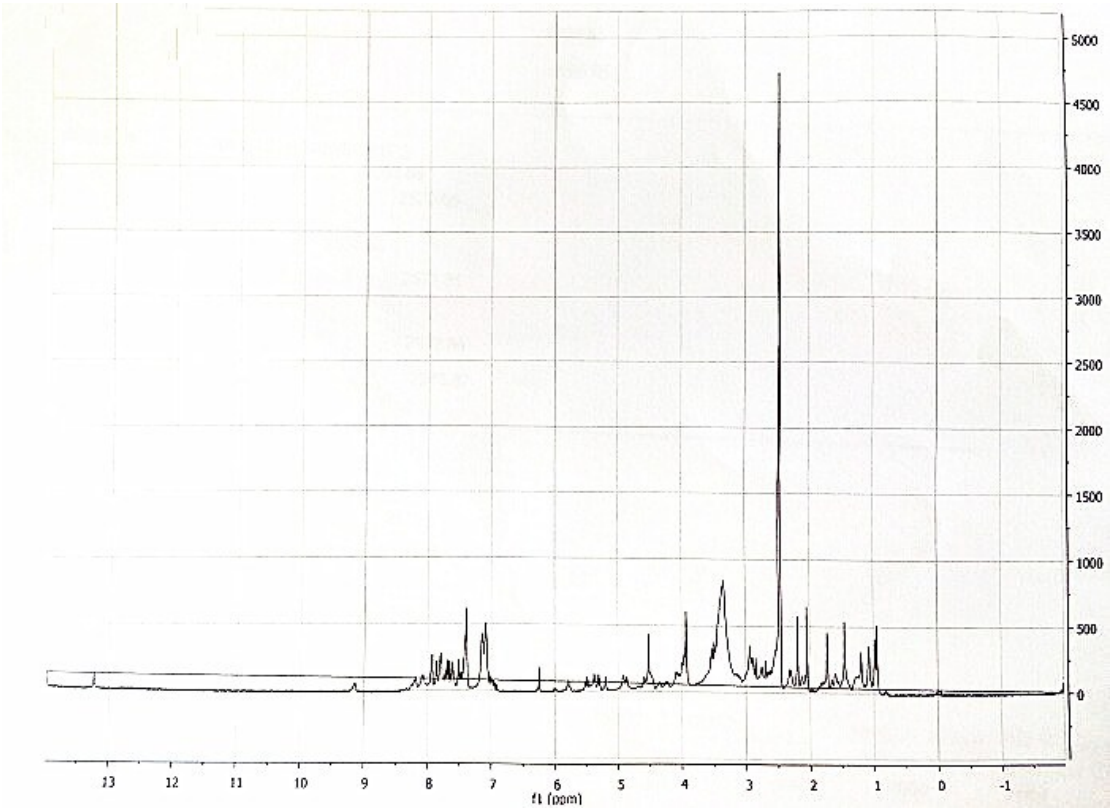


Figure S15 ^{1}H NMR spectrum of NapFFK (taxol) C (dox) Yp **(8)**.

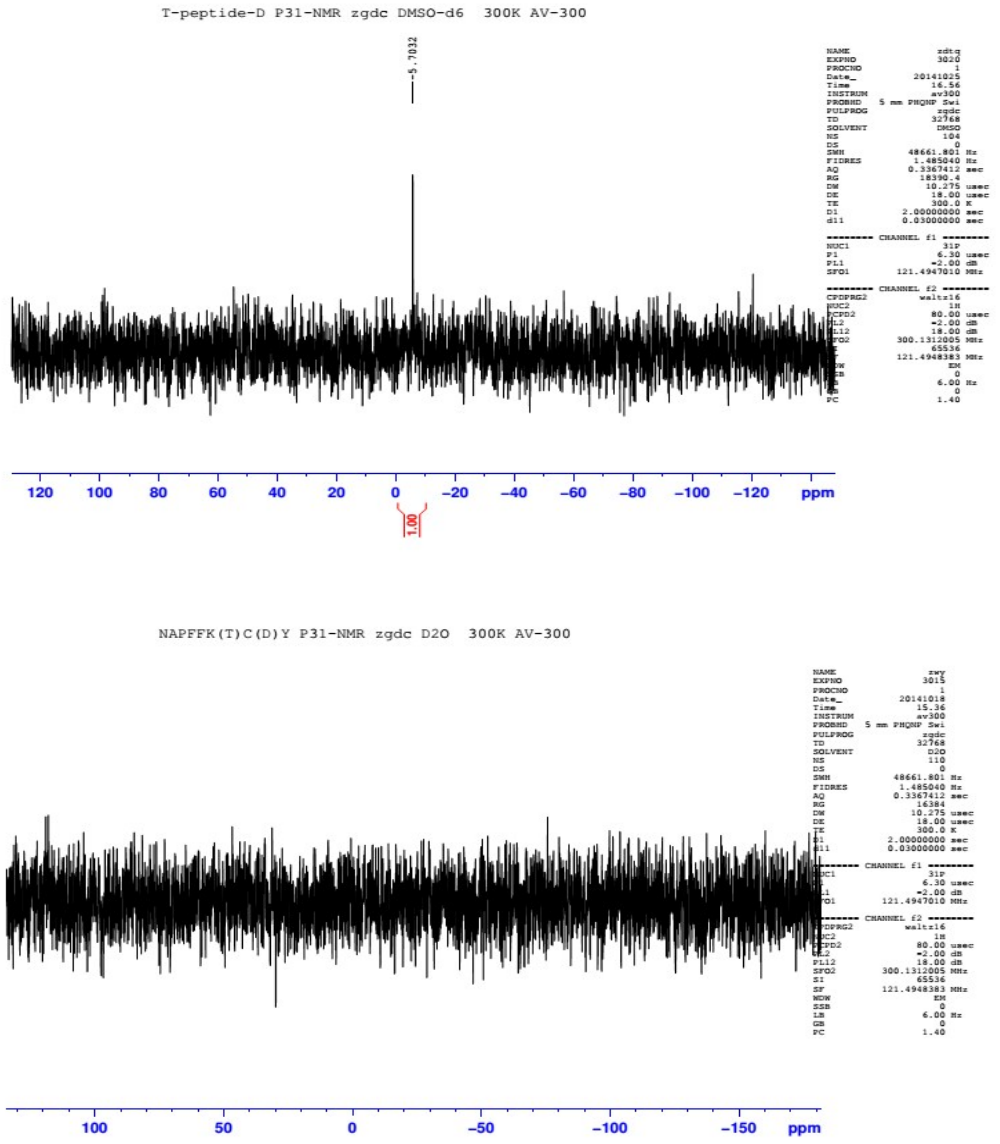


Figure S16 The ^{31}P NMR spectra of **8a** before and **8b** after the addition of ALP.

3. The morphological characteristics of hydrogels

TEM is often used to characterize the structure of self-assembled hydrogels. several drops of diluted hydrogel of **1b** and **8b** at 1.0wt% were applied to a carbon-coated grid and performed on a JEOL JEM-2010f electron microscope at 220 kV. When added the ALP, the morphology of the hydrogel at different time were characterized in Figure S16. The Scanning Electron Microscope (SEM) sample powder was placed in copper mesh and the images were obtained by S-4800 Scanning Electron Microscope.

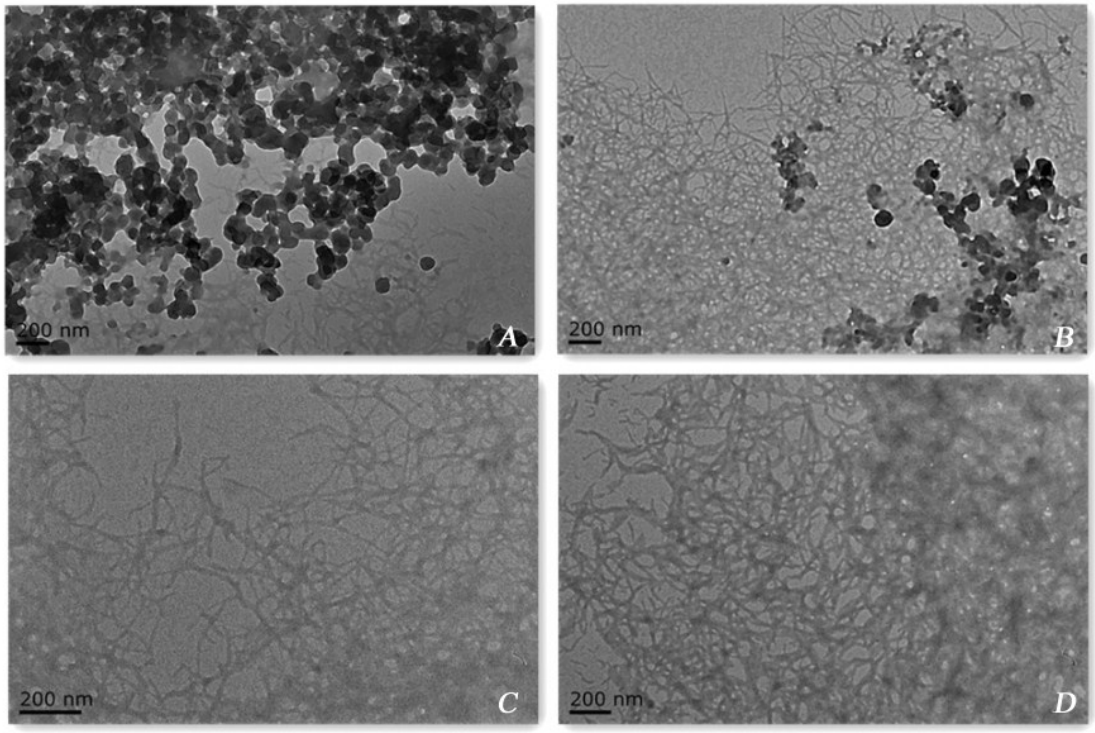


Figure S17 TEM image of solution 8a (2.0wt%) after addition of ALP in 5 minutes (A) , 10 minutes (B) , 20 minutes (C) and 40 minutes (D).

4. Rheological test

Rheology experiments were conducted on a Thermo RheoStress 600 to examine the viscoelastic properties of the hydrogel **8b**. The dynamic frequency sweep was conducted to measure the frequency response of the hydrogel in the region of 0.1-100 rad/s at the strain of 1%. Dynamic strain sweep was performed to measure the linear viscoelastic region of hydrogel at the frequency of 6.28 rad/s and temperature of 37℃.Upon the hydrogel was subjected a shear of 1000 s^{-1} for 0.5 minute, dynamic time sweep was used to ensure the recovery of hydrogel by monitoring the storage (G') and loss (G'') modulus at frequency of 6.28 rad/s and strain of 1%.

5. In vitro drug release

To the solution of 0.2 ml distilled water containing 2 mg of **8a** at pH=7.6, hydrogel of **8b** formed when alkaline phosphatase (38U/mL) was added. Then 0.25 ml of fresh PBS buffer solution (100 mM, pH=7.4) was added onto the surface of the gel. At the end of every hour, 0.2 ml supernatant PBS solution was taken out and another 0.2 ml fresh PBS solution was added back to the gel. HPLC (Agilent 1200) was applied to monitor

the release of Taxol and Dox. Mobile phase system is methanol: water (adjust pH to 3.0 with phosphoric acid) 80:20, column temperature is 40 ℃ and the detection wavelength is 230nm, the experiment was conducted for three parallel tests.

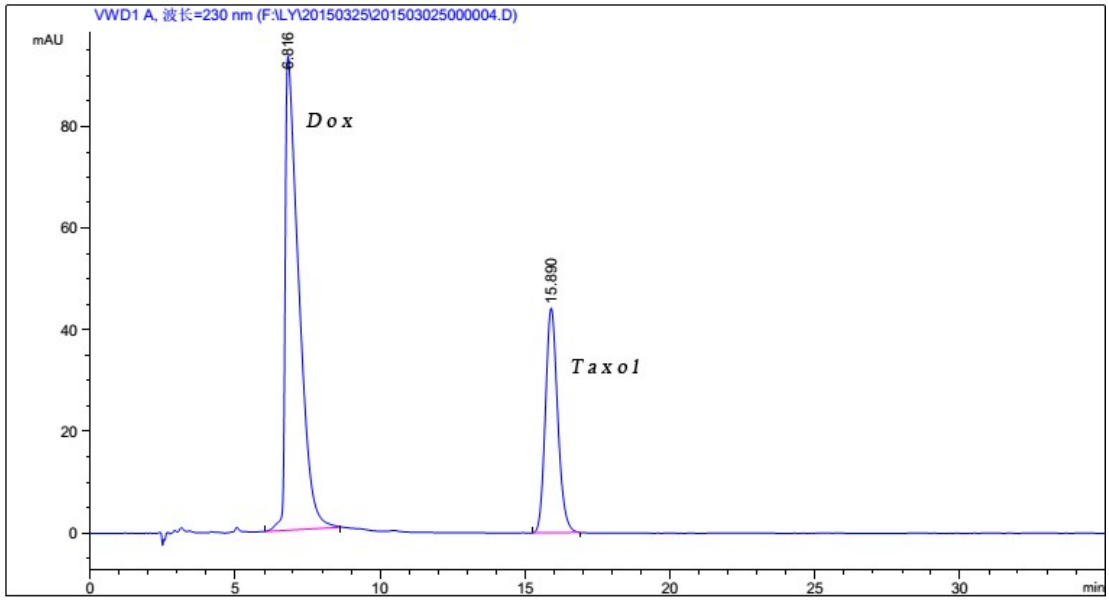


Figure S18 The separation profile of Dox and Taxol in HPLC

6. Cytotoxicity measurement

The cytotoxicity was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Biosharp) assay. The MCF-7 cells (ATCC, America) were incubated in medium of RPMI-1640, containing 10% fetal bovine serum at 37℃ in 5% CO_2. Cells in 100μl of medium volume was seeded in each well on the 96-well plate at density of 2,000 cells/well and incubated for 24 h to allow the attachment of cells to well surface. Then 100 μl of the solutions containing different samples (peptide, peptide hydrogel, Dox-peptide, Taxol-peptide, Dox-peptide-Taxol, Dox-peptide-Taxol hydrogel) were added into the medium and calculated for 48 h, then replaced the medium with 20 μl MTT reagent (5 mg/mL) and added DMSO (100uL/well) to dissolve the formazan crystals after 4 hours. Using a microplate reader (Bio-RAD, America) to measure the density of solution at 490nm. The experiment was conducted for three times and cells without treatment as control group. The concentrations of the compounds when 50% of cells viability was recorded represented the IC50 values of the compounds.

The cell viability percent (%) = OD sample / OD control * 100%

7. Application for potential Intracellular Imaging

The Dox-peptide-Taxol hydrogel was prepared to monitor the targeting and anticancer effect due to the red fluorescence of the Doxorubicin. The MAD-MB-231 cells was used for imaging. First, seed the MAD-MB-231 in 24 well microtiter plates for 24 hours, then added the hydrogel of **8b** with different concentrations to the cells and incubated for 1 hours at 37℃ and 5% CO_2. The cell without the hydrogel was as control group. Then imaged on a confocal microscope (Olympus IX71, Japan**)**.

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