Electronic Supplementary Material (ESI) for ChemComm.

Supporting information

Materials and general methods:

Materials: All Fmoc-amino acids and 2-chlorotrityl chloride resin were purchased from GL Biochem (Shanghai, China). 2-(1H-benzotriazol-1-yl)-1,1,3,3 –tetramethyluronium hexafluorophosph- ate (HBTU), 1-hydroxy-benzotriazole monohydrate (HOBT), trifluoroacetic acid, 4-dimethylamino-pyridine, diisopropylethylamine (DIPEA), HCPT, Hoechst 33342 was obtained commercially from Aladdin Reagent Corporation (Shanghai, China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) was obtained from Biosharp Company (Hefei, China). Docetaxel(DTX) was obtained commercially from Dalian Meilun Biotech Company, Ltd. (Dalian, China). Doxorubicin(Dox) was purchased from Jingge Biotech Company, Ltd. The human-derived liver cell L02, the human prostate cancer cell DU145 and PC-3 was purchased from American Type Culture Collection (ATCC, China's district general agent, Beijing, China).

Peptide synthesis: The peptides were synthesized via solid phase peptide synthesis method (SPPS). After cleavage from the resin and deprotection of the side groups and N-terminus using the TFA only, the peptides were collected in cold ether, centrifuged and freeze-dried. The obtained crude products were purified by reverse phase HPLC. MS, ^{1}H NMR, HPLC were used to verify the peptide structure and value their purity.

The synthetic routes of these forky peptides were illustrated as Fig.S1.

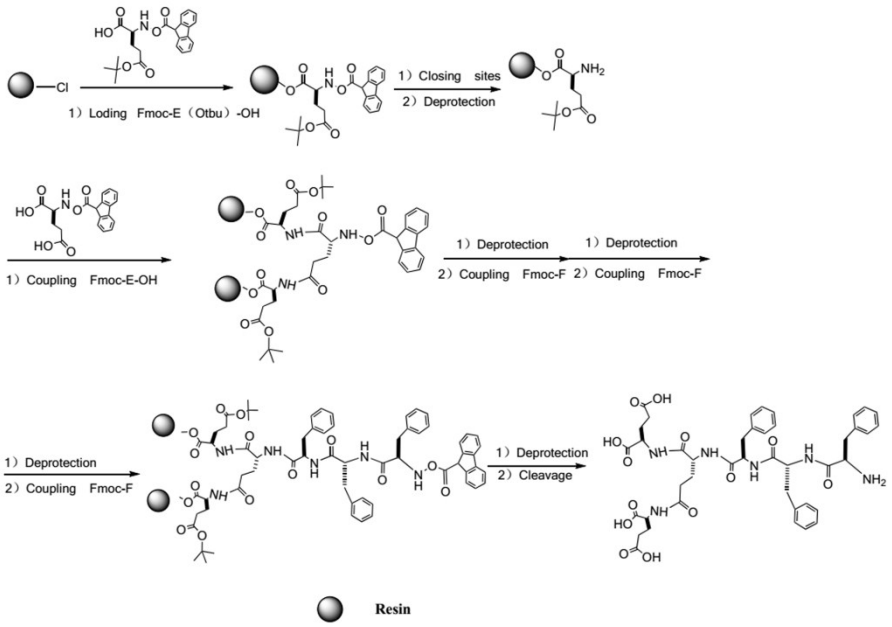

Fig.S1. Schematic diagram of the synthetic routes.

The purification of E_3F_3 peptide was handled as follow.

The purification was performed on liquid chromatograph(Shimadzu LC-20AR). Chromatographic conditions：The semi-preparative Chromatographic column, Shim-pack GIS C18 column(250×20mm i.d.,10um).Mobile phase: The mobile phase consisted of two eluents, phase A was acetonitrile, phase B was 0.1% TFA(trifluoroacetic acid) solution. Fixed mobile phase ratio was 37.5:62.5.Injection volume: 2 mL. Flow rate: 10.0 mL/min. T=35 °C. The DAD monitoring

wavelength was 260 nm. The collection time was 6.5 to 7.5 min according to semi-preparative chromatogram(Fig.S2). The elution fluid was concentrated by vacuum rotary evaporation and freeze drying.

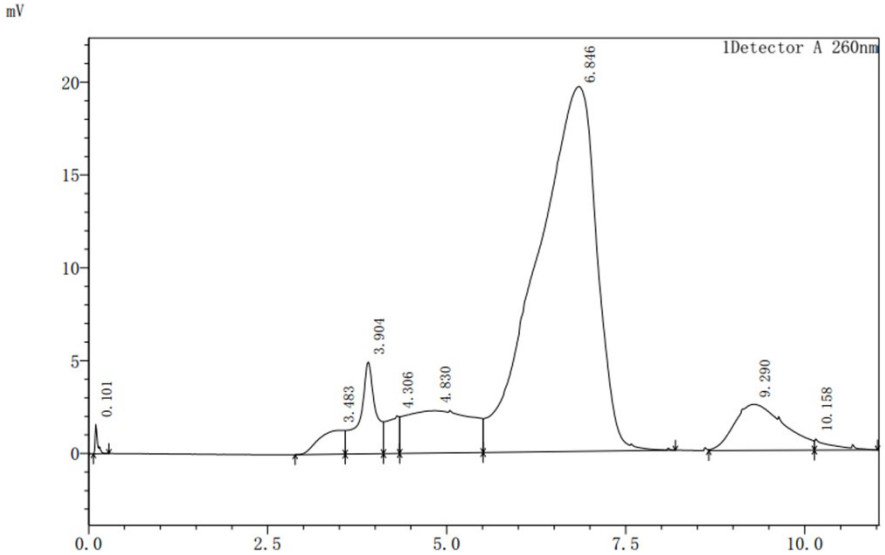

Fig.S2. The chromatogram of E_3F_3 peptide for semi-preparative operation.

HPLC(Shimadzu LC-20A) was used to confirm operating conditions of the purification and value peptide purity, the chromatogram of E_3F_3 peptide before and after purificating was illustrated as Fig.S3-4.

Chromatographic conditions：Chromatographic column: C8 ODS Hypersil column (250×4.6 mm, i.d., 5μm).Mobile phase: The mobile phase consisted of two eluents, phase A was acetonitrile, phase B was. 0.1% TFA(trifluoroacetic acid) solution. Fixed mobile phase ratio was 37.5:62.5.Injection volume: 10 μL. Flow rate: 1.0 mL/min. T=35 °C. The DAD monitoring wavelength was 254 nm.

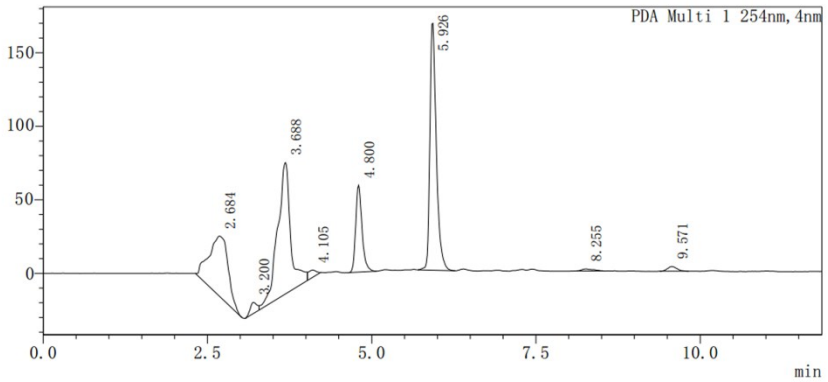

Fig.S3. The chromatogram of E_3F_3 peptide before purificating.

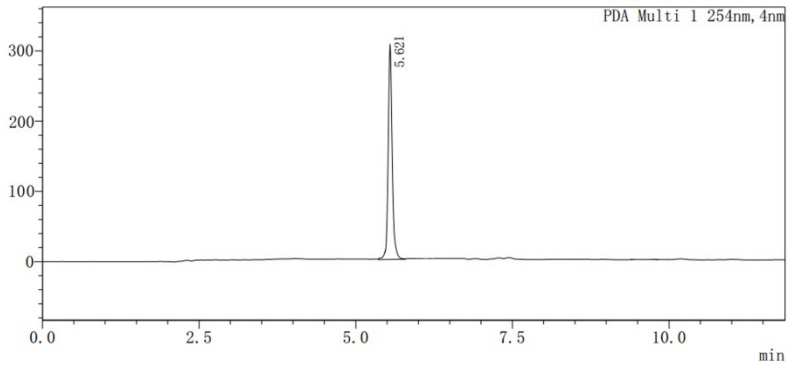

Fig.S4. The chromatogram of pure E_3F_3 peptide.

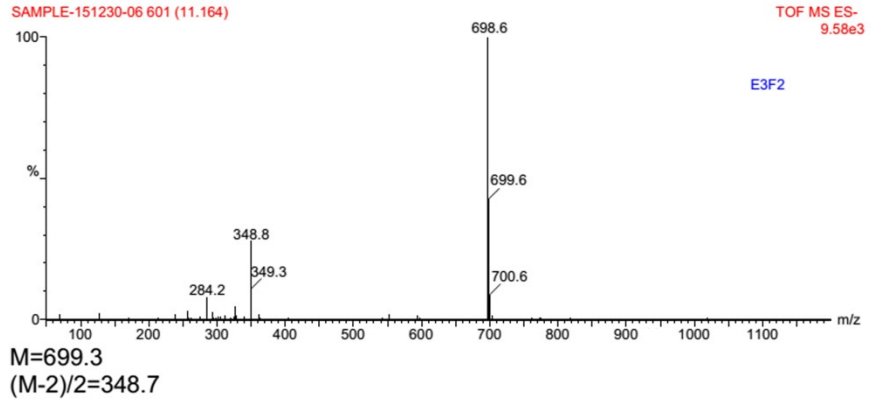

Fig.S5. MS of E_3F_2 peptide compound.

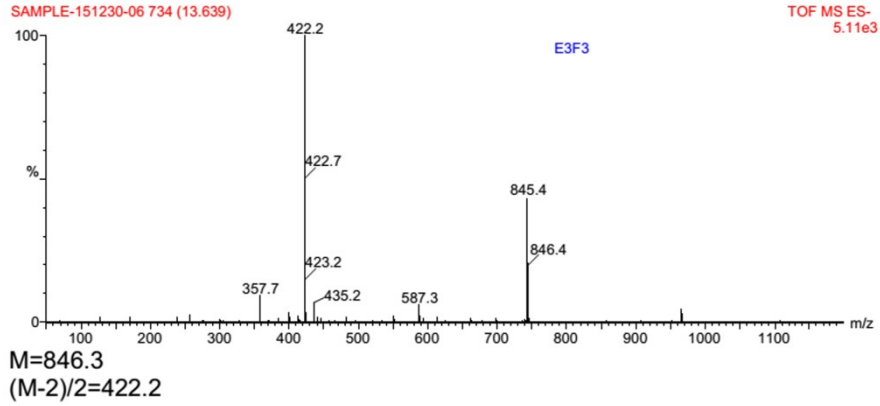

Fig.S6. MS of E_3F_3 peptide compound.

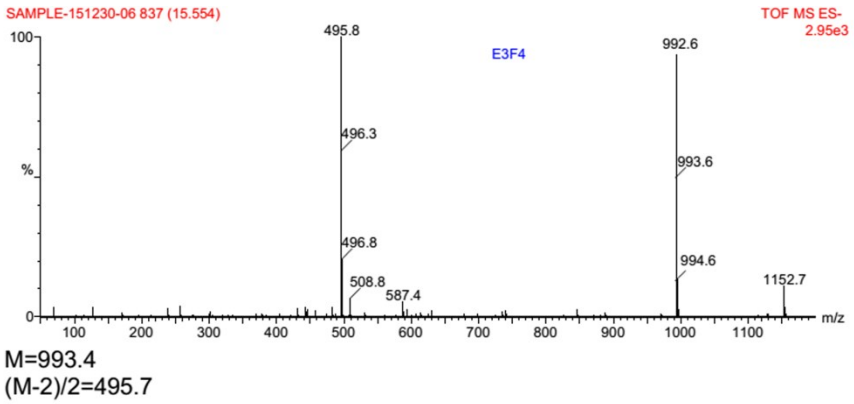

Fig.S7. MS of E_3F_4 peptide compound.

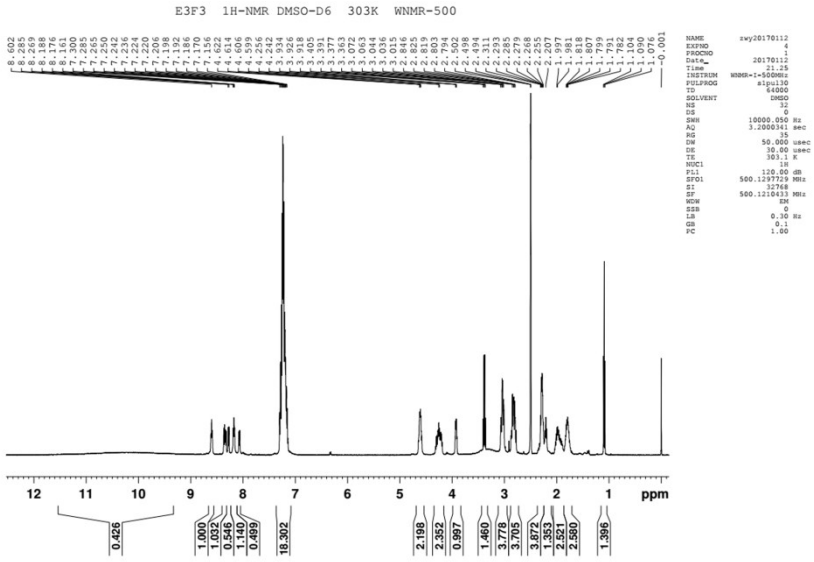

Fig.S8. ^{1}H-NMR of E_3F_3 peptide compound.

TOF-MS of E_3F_2: m/z 698.6 [M-H], 348.8 [(M-2H)/2]. TOF-MS of E_3F_3: m/z 845.4 [M-H], 422.2 [(M-2H)/2]. TOF-MS of E_3F_4: m/z 992.6 [M-H], 495.8 [(M-2H)/2].^{1}HNMR (500 MHz, DMSO) of E_3F_3: δ 8.615~8.587 (m, 2H), 8.369~8.064 (m, 5H), 7.300~7.156 (m, 15H), 4.645~4.581 (m, 2H), 4.305~4.191 (m, 3H), 3.943~3.909 (m, H), 3.405~3.363 (m, 2H), 3.072~3.015 (m, 2H), 2.913~2.777 (m, 2H), 2.329~2.255 (m, 4H), 2.234~2.191 (m, 2H), 2.023~1.888 (m, 4H), 1.835~1.752 (m, 2H).

Preparation of hydrogels.

The E_3Fn peptide has a slight difficult to dissolve in the phosphate buffered saline (PBS) solution (pH = 7.4). In order to improve the solubility property, a small amount of 0.1M sodium carbonate solution was dropped in. The above solution was incubated at room temperature overnight, then the zinc ions solution was added in to trigger the formation of the translucent hydrogel. The apparent minimum gelation concentration of each forky peptide was confirmed by vial inversion.

Gelling properties：

A series peptide solution，at different concentration level, was prepared and placed overnight, then zinc ions(ZIs) were added into stepwise. The apparent minimum gelation concentration(MGC) of each dendron peptide was confirmed by vial inversion and recorded (Tab.S1).

We investigated the rough ion concentration in plasma through summarizing correlational research literature, and recorded as follows. 1% E_3F_3 solution was prepared and various divalent cation was added into, for determining of the ions trigger concentration (Tab.S2; Fig.S9b).

Tab.S1 The MCG of different E_3Fn peptides and the trigger concentration of zinc ion and calcium ion.

Peptide category	E_3F_2	E_3F_3	E_3F_4
MCG	0.75%	0.8%	1.5%
Trigger concentration of ZIs(μ g/ml)	221.2	251.5	622.8

Tab.S2 The trigger concentration of different divalent cation for E_3F_3 peptide.

Category	Zn	Ca	Mg	Cu	Ni	Fe
Plasma concentration(μ g/ml)	0.83	47.20	12.72	1.36	0.0005 9	0.92
Trigger concentration(μ g/ml)	268.2	372.0	311.2	451.2	193.7	720.5

The minimum time needed for gelation (MTG) were confirmed through a series parallel experiments, in which we recorded the gelling time after adding 5Mm zinc ion into 1% E_3F_3 peptide solution and one minute shaking. We confirmed the time by observing inversional vial every 30 seconds(Tab.S3).

1ml whole blood was collected from the heart of healthy SD rat(SPF), 4wt% disodium citrate was chose as anticoagulation and added into at the ratio 1:16. E_3F_3 solution was mixed with the whole blood of rat，the concentration of peptide was 2wt%.After one minute shaking and ten minute standing, the vial was inclined, the mixture was mobile (Fig.S9a).

Tab.S3 The MTG for E_3F_3 peptide.

Serial number	1	2	3	4	5	average	SD
MTG(min)	4.5	5.5	4	4.5	4	4.7	0.57

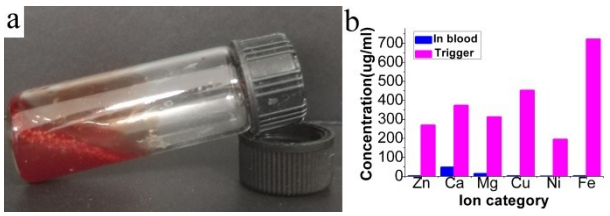

Fig.S9 a:the image of peptide solution mixed with shole blood; b:The ion concentration of blood in contrast with the trigger concentration of various metal ion for 1% E_3F_3 peptide .

Gelling test in vivo：

To verify the hydrogel formation could happen at the prostate, gelling test in vivo was conducted, using rat as experiment animal. Doxorubicin, a carmine anticancer-drugs, was mixed into 1wt% E3F3 solution for facilitating visual observation of the distinction between hydrogel and normal prostate tissue. The concentration of doxorubicin was 0.4mg/ml consistently. All rats were adult male, SD (SPF), weight was about 300~350g. 10wt% chloral hydrate was used as anesthetic. After the anesthesia through intraperitoneal injecting, both the solution of doxorubicin and the E_3F_3 peptide solution was injected into ventral page of prostate. Each ventral page were injected at two sites, the amount was 50 μ l respectively. 4 hours after the injection, the rat was executed and excised its prostate immediately, then the prostate was observed by stereomicroscope（Olympus SZX7, Japan）.

Preparation of hydrogels triggered by different concentration ZIs:

2mg E_3F_3 peptide was dissolve in 750 μL H_2O, 250 μL 0.1M sodium carbonate solution was dropped in, the above solution was incubated at room temperature overnight. The concentration of peptide is ~2.4mM. Then the solution was diluted with H_2O, a series zinc solution was added into. The final hydrogels was 1%W(~ 1.2 Mm), with a series mole ratio of peptide vs ZIs. The mole ratio is 3:1, 2.5:1, 2:1, 1.5:1, 1:1 and 0.5:1 respectively.

Fluorescence spectroscopy: Fluorescence emission measurements were conducted in a FL-4600 spectrofluorimeter (Hitachi, Japan). To investigate the aromatic stacking in the formed hydrogel, emission spectra were recorded from 285 nm to 550 nm with an excitation wavelength of 275 nm. In this experiment, we prepared hexapeptide saturated aqueous solution and diluted hydrogel solution (0.5 mg/mL). The interaction of peptide molecules was testified also by fluorescence spectrophotometry. In both experiments, the excitation and emission slits were set 5 nm with an assay volume of 1.0 mL.

Circular dichroism (CD)**:** The data were collected at 25 °C on a Jasco (Tokyo, Japan) J-810 CD spectropolarimeter. The concentration of peptide was 0.3 mg/mL, 1.5 wt% hydrogels were diluted 50 times. Data was reported from 250 nm to 190 nm. Spectra were corrected by subtraction of the doubly distilled water which was the same as the solvent of the samples.

Transmission electron microscopy: 5 μL of diluted 1.0 wt% hydrogel was applied to a carbon-coated grid and excess water was blotted away with filter paper after 1 min. Afterwards, the grids were left to dry in ambient conditions for 30 min at least. Transmission electron microscopy (TEM) samples were performed on a JEM-2100 electron microscope (Japan) operating at 220 KV.

Scan electron microscopy: the samples powders freeze-dried previously of 1.0 wt% hydrogel was deposited on a copper stub and the images were obtained by S-4800 Scanning Electron Microscope (Japan).

Oscillatory rheology: Rheological tests including dynamic time sweep, dynamic frequency sweep, and dynamic strain sweep were performed on a RheoStress 600 (Thermo) instrument using 60 mm parallel plates at a gap of 0.5 mm. The tests were conducted to record the change between storage (G′) and loss (G″) modulus at 37 °C. Firstly, the gel was carried out dynamic time sweep with a constant frequency of 6.28 rad/s and constant strain value of 1% within 30 min. During the dynamic time sweep, hydrogels were firstly transformed to solution state by vortex vibration(5min), then recovery of hydrogels was examined by recording viscoelastic properties of hydrogels with the increase of time. Then, the gel was characterized by the mode of dynamic frequency sweep within the region of 0.1-100 rad/s at the strain of 1%. Next, the gel was conducted dynamic strain sweep test, in the strain region of 0.1-100% at the frequency of 6.28 rad/s. Finally, the circle sweep was conducted, the low level of shear strain was 2% and the high level was 50%.

Preparation of hydrogels loaded DTX for drug realease.

The hydrogels with identical peptide concentrations 0.9%、1.2% and 1.5% were prepared and loaded with DTX severally. 9mg、12mg and 15mg E_3F_3 peptide was dissolve in 600 μL phosphate buffered saline (PBS) solution (pH = 7.4) respectively, in order to improve the solubility property, 200 μL 0.1M sodium carbonate solution was dropped in, then 7.5 μL、10 μL and 12.5 μL DTX solution at 20mg/mL was added into correspondingly. Finally the PBS was supplemented and the total volume of each solution was 950 μL. The above solution was

incubated at room temperature overnight, then 50 μL zinc ions solution(0.1M) was added in to trigger the formation of the translucent hydrogel. The mass ratio of DTX and peptide was 1 to 60.

Determination the content of DTX:

HPLC(Agilent 1200) was used to Determination the content of DTX Chromatographic conditions：Chromatographic column: C18 ODS Hypersil column (250×4.6 mm, i.d., 5 μm).Mobile phase: The mobile phase consisted of two eluents, phase A was acetonitrile, phase B was. deionized water. Fixed mobile phase ratio was 50:50. Injection volume:10μL. Flow rate: 1.0 mL/min. T=35 °C. The UVD monitoring wavelength was 227 nm.

The chromatogram of pure DTX, E_3F_3 peptide and hydrogel loaded DTX was illustrated as Fig.S10-12. The experiment was carried out three times at least. The calibration curve of DTX was illustrated as Fig.S13. The equation is $A=5.815*C_{DTX}-37.159$, R was 0.9993.

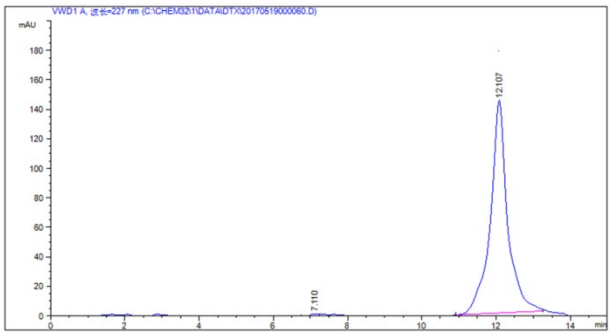

Fig.S10. The chromatogram of pure HCPT

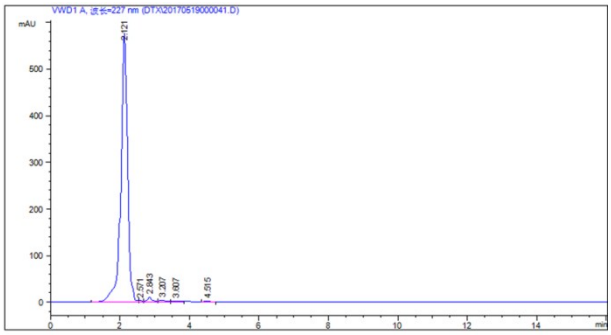

Fig.S11. The chromatogram of E_3F_3 peptide compound.

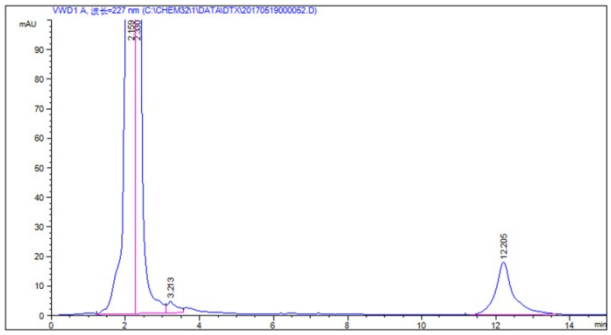

Fig.S12 The chromatogram of E_3F_3 peptide hydrogel loaded HCPT.

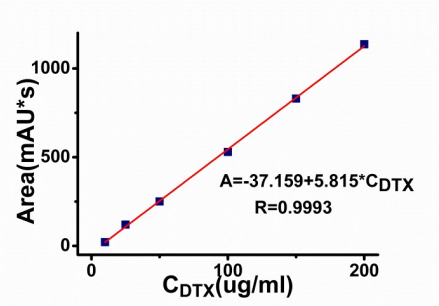

Fig.S13. The calibration curve of DTX.

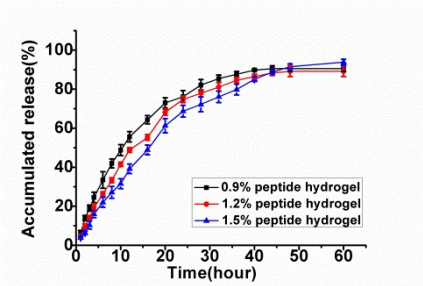

Fig.S14. The cumulative release of DTX from E3F3 hydrogels. Mean±SD (n=3).

Release mechanism: To provide an explanation about the conceivable mechanism of the DTX release from the hydrogels, the data were analyzed fitting to the following empirical equations:

Model: Ritger-Peppas equation

$M_t / M_\infty = k \cdot t^n$

Where M_t/M_∞ is fractional drug release, M_t is the amount of drug released at time t, M_∞ is the maximum amount of drug released at time ∞, t is the release time, k is a rate constant of kinetic release, and n is the diffusion exponent, characteristic of the drug release mechanism. For n< 0.5, it indicates that the drug release follows the Fickian diffusion, whereas the non Fickian drug release process has a value of n between 0.5 and 1.

Tab. S4. The calculated data of DTX hydrogel in drug release experiment of different peptide concentrations.

	Ritger-Peppas		
gel concentration	k	R^2	n
0.9 wt%	0.1043	0.9760	0.6232
1.2 wt%	0.0682	0.9802	0.7342
1.5 wt%	0.0442	0.9824	0.8416

Cell culture: The human-derived liver cell L02, the human prostate cancer cell DU-145 and PC-3 were grown in RPMI-1640 supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) at 37 °C in a 95% humidified air, 5% CO_2 atmosphere.

Preparation of hydrogels loaded DTX with trigger ions（Zn）:

24mg E_3F_3 peptide was dissolve in 750 μL phosphate buffered saline (PBS) solution (pH = 7.4), 250 μL 0.1M sodium carbonate solution was dropped in, 400 μL DTX solution at 1 mg/mL was

added into correspondingly. Then 480 μ L PBS was added into. The above solution was incubated at room temperature overnight, then 120 μ L zinc ions solution(0.1M) was added in to trigger the formation of the translucent hydrogel.

E_3F_3 peptide in contact with L02 cells: 500 μL of single cell suspension ($1.5x10^4$/mL) was shifted in 24-well plate and cultured in an incubator for 24 hours. Thereafter, 500μg/ml E_3F_3 peptide hydrogel was prepared as samples. The next day, 500 μL of each sample was added into each well and cultured for 24 or 48 hours with the phosphate buffered saline as control. After that, 0.5 mL of fresh PBS buffer was used to replace the medium for two times. Ultimately, the light microscope images were obtained by a inverted fluorescence microscope (Olympus IX71, Japan).

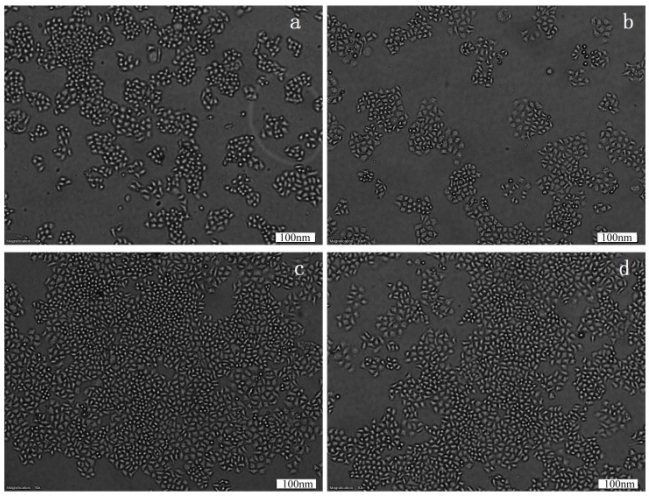

Fig.S15. Images of L_{02} cells affected by fresh PBS buffer for 24h (a) and 48h(c), the 200 μg/ml peptides for 24h (b) and 48h(d).

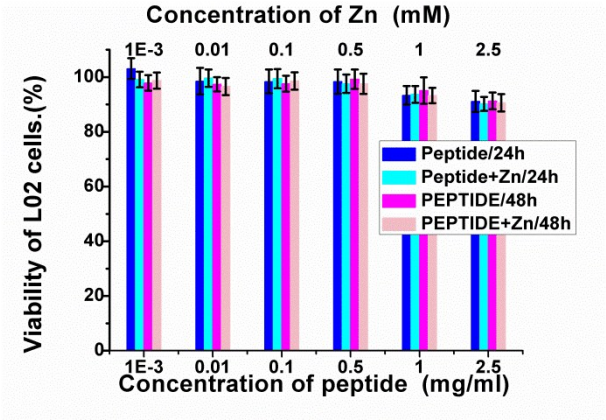

Fig.S16. The cell viability of the forky-peptide from low to high concentration.

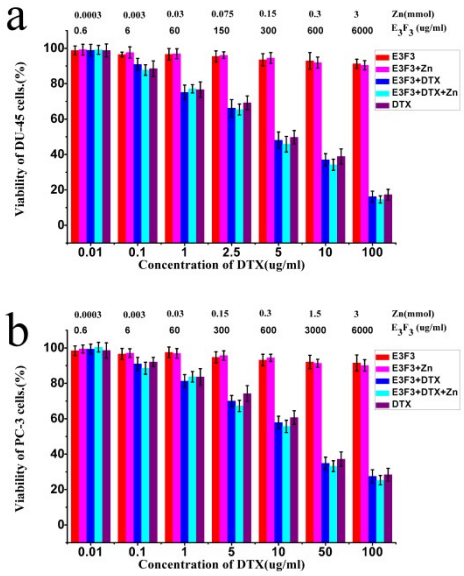

Fig.S17. The cell viability of the forky-peptide hydrogel with and without ZIs, the DTX-loaded hydrogel with and without ZIs, and free DTX low to high concentration. (a DU-145;b PC-3. Mean±SD(n=6) .).

In vitro anticancer efficacy and cytotoxicities evaluation:

100 μL of single cell suspension ($5x10^4$/mL) was injected into 96-well plate and cultured in an incubator for 24 hours. Subsequently, the same volume of the E_3F_3 peptide hydrogel ,the free drug and the drug-loaded hydrogel with several gradient concentrations were added to each well with the phosphate buffered saline as control (five parallel well for each concentration) for 24 hours. After that, the media was washed twice by 200 μL of fresh culture medium. 20 μL of MTT solution (5 mg/mL) was added to the plate before incubated for 4 hours at 37 °C. Subsequently, the MTT solution was drained from the plate and 200μl of DMSO was added to each well to dissolve the formazan crystals. The density of viable cells was then determined by measuring the optical density at 570nm (Bio-RAD, America) after concussion for 10 min.

1000 μL of single cell suspension ($1.5x10^4$/mL) was shifted in 6-well plate and cultured in an incubator for 24 hours. The E_3F_3 peptide solution with confirmed concentration 600 μg/ml, the solution of free drug and the drug-loaded hydrogel with a consistent DTX concentration 10 μg/ml were prepared as sample. Hereafter, 1000 μL of each sample was added into each well and cultured for 24 hours. After that, 1μL Hoechst 33342 solution, the concentration was 10mg/mL, was added into each well. Then 1mL of culture medium was used to replace and cleanout for three times. Ultimately, the fluorescent microscope images were obtained by a inverted fluorescence microscope (Olympus IX71, Japan).

The apoptotic percentages quantified with Hoechst staining and fluorescent microscopy.

The apoptotic percentages for drug-treated DU-145 and PC-3 cells were then quantified with Hoechst staining and fluorescent microscopy (Olympus IX71, Japan). Those cells with densified or granular fluorescence were counted be apoptotic, and the ratio of apoptotic cells was normalized against total number of cells. Hydrogel itself didn't induce

apoptosis for both DU-145 and PC-3 cells (Fig.S16 9a,9d). The treatment of free DTX (10 μg/ml, ~24 hours) caused ~46.0±3.9% and 51.3±2.8% of DU-145 and PC-3 cells undergoing apoptosis respectively(Fig.S16g). In contrast, at the same drug concentration, the treatment of DTX-loaded hydrogels led to ~ 51.9±3.2% and 58.0±3.8% of apoptotic cells for both cell lines. The differences of percentages of apoptotic cells between free DTX and DTX hydrogel were significant ($P<0.05$), which is consistent with the data of MTT assays and further confirmed the high potency of DTX in the peptide hydrogel.

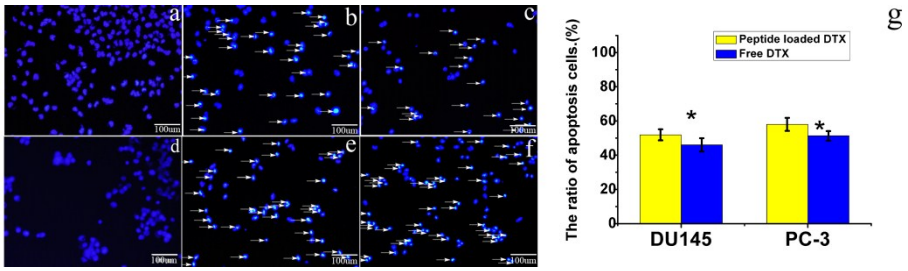

Fig.S18 The Inverted fluorescence image of DU-145 cells and PC- cells.(a-c: the DU-145 cells incubated with E_3F_3 peptides, hydrogel loaded DTX and free DTX respectively; d-f: the PC-3 cells incubated with E_3F_3 peptides, hydrogel loaded DTX and free DTX respectively; g:the percentages of apoptotic cells after drug treatment. The concentration of peptide was 600 μg/ml, and DTX was 10 μg/ml consistently. The arrows labelled apoptotic cell. Mean±SD (n=5) .*, $P<0.05$.

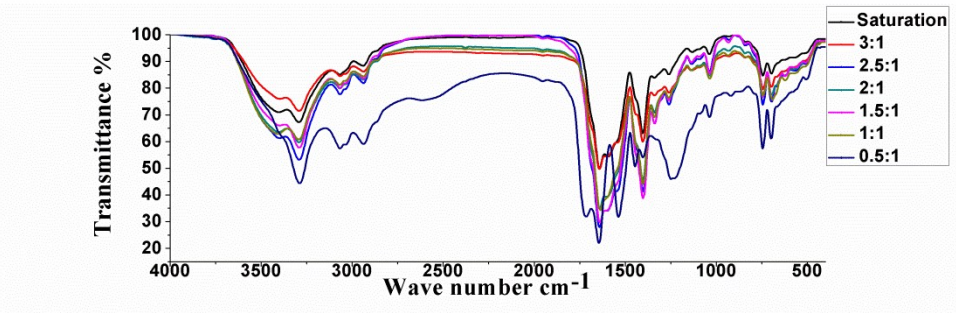

Fig.S19 IR of different hydrogels