Metal ion and light sequentially induced sol-gel-sol transition of a responsive peptide hydrogel

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Supporting Information

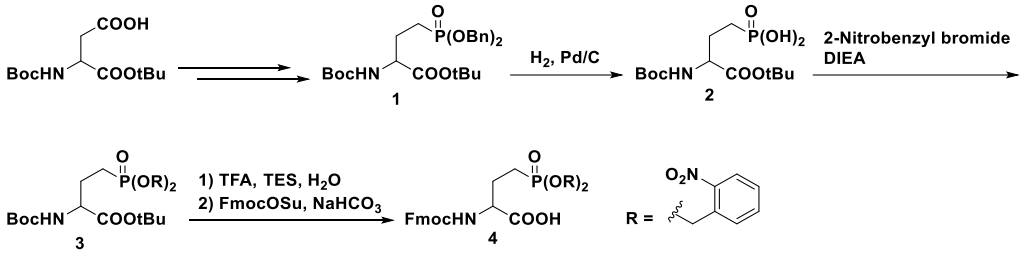
1. General Information

L-Boc-Asp-OtBu, *N*-Fluorenyl-9-methoxycarbonyl (Fmoc) protected L-amino acids (Fmoc-Phe-OH, Fmoc-Ser(tBu)-OH, and Fmoc-Ser(HPO$_3$Bzl)-OH) and 2-chlorotrityl chloride resin were purchased from GL Biochem (Shanghai) Ltd. (China) and used as received. All other reagents and solvents were purchased from Alfa Aesar or ACROS (USA) and used directly.

Fmoc-pSer^C(OBn)-OH was synthesized by Kang's method[1]. The synthesis of Fmoc-pSer^C(oNB)-OH is in **Supporting Information 2**

ESI-MS spectra were obtained using a Thermo quadrupole mass spectrometer. Analytical HPLC of peptides were performed on a Shimadzu LC-2010A HT HPLC system with an YMC pack C8 column (5 μm, 4.6 x 150 mm), with 0.6% TFA in water (**A**) and 80% acetonitrile, 0.6% TFA in water (**B**) as eluents, 45% B to 100% B in 25 min. Preparative HPLC of peptides were performed on a Shimadzu LC-6AD 230V ASSY HPLC system with an YMC pack C18 column (5 μm, 20 x 250 mm) using the same eluents as the analytical HPLC.

2. Synthetic route of Fmoc-pSer^C(oNB)-OH (4)



Scheme S1 Synthetic route of Fmoc-pSer^C(oNB)-OH (**4**).

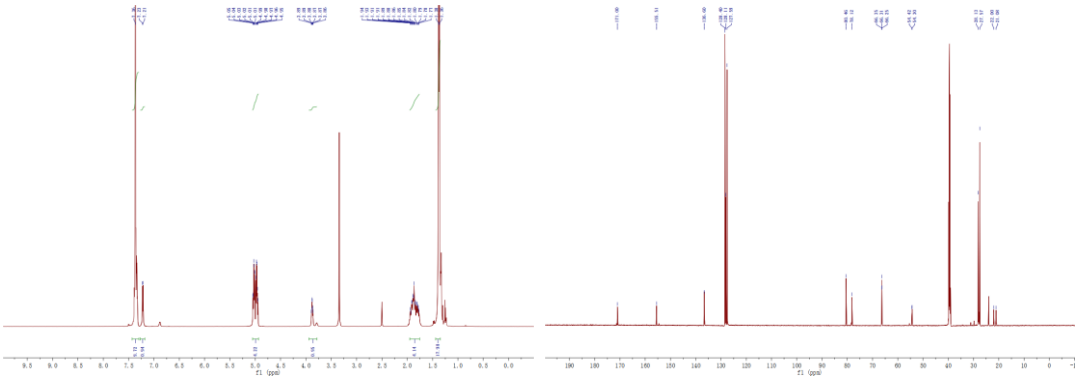
1 was synthesized as Kang's method[1].

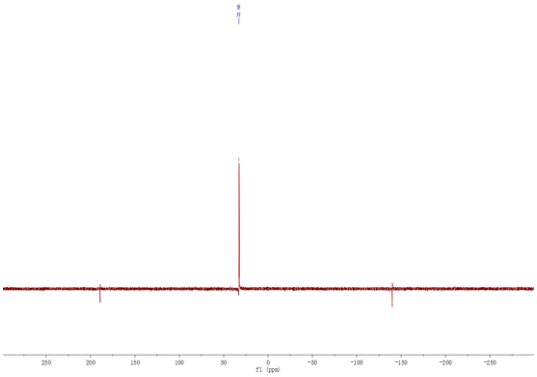
^{1}H NMR (600 MHz, DMSO-D6) δ 7.36 (s, 10H, C$_6$**H$_5$**), 7.22 (d, J = 7.8 Hz, 1H, N**H**), 5.05 – 4.94 (m, 4H, C**H$_2$**C$_6$H$_5$), 3.88 (td, J = 8.2, 4.4 Hz, 1H, α-C**H**), 1.95 – 1.76 (m, 4H, β-C**H$_2$** and γ-C**H$_2$**), 1.37 (d, J = 9.1 Hz, 18H, C$_4$**H$_9$**).

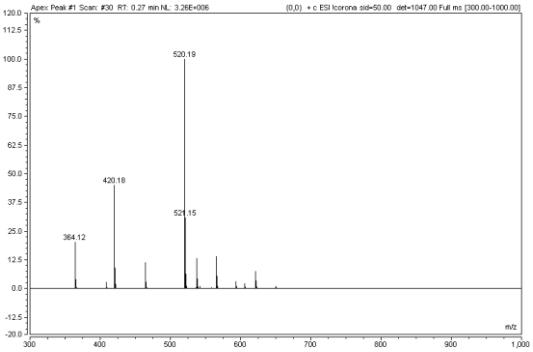
^{13}C NMR (151 MHz, DMSO-D6) δ 171.00, 155.51, 136.60, 128.40, 128.11, 127.59, 80.46, 78.12, 66.31, 54.36, 28.13, 27.57, 22.00, 21.08.

^{31}P NMR (243 MHz, DMSO-D6) δ 32.66.

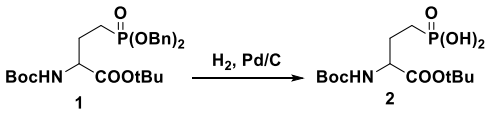
ESI-MS: $[M+H^+]^+$ calcd for $C_{27}H_{39}NO_7P^+$ 520.25, found 520.19.





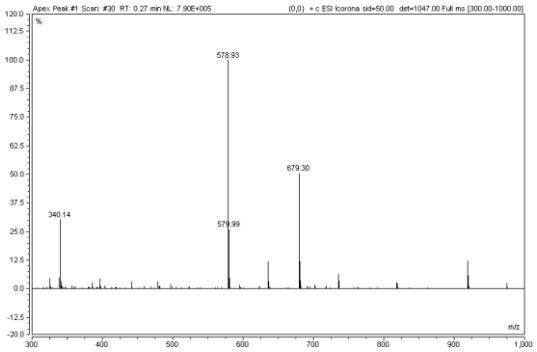


2.1. Synthesis of (2)

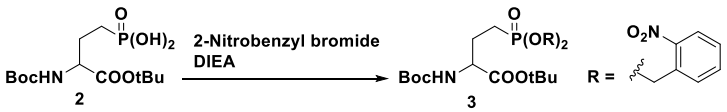


1 (2.60 g, 5.0 mmol) was dissolved in methanol (100 mL) and palladium (5% loaded on activate carbon) was added in the solution in nitrogen atmosphere. Nitrogen was replaced by hydrogen and the resultant mixture was stirred at room temperature for 24 h. The resultant mixture was filtered and concentrated in vacuo to afford **2**. Crude product **2** (1.61 g, 4.75 mmol, 95%) was obtained as a white solid and it can be directly used for the next step.

ESI-MS: $[M+H^+]^+$ calcd for $C_{13}H_{27}NO_7P^+$ 340.15, found 340.14.

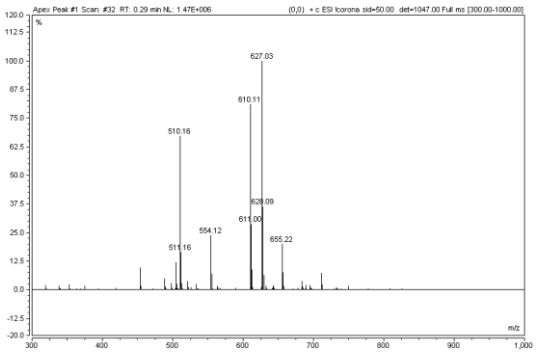


2.2. Synthesis of (3)

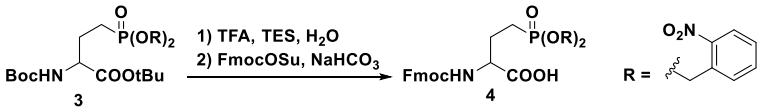


To a stirred solution of **2** (1.61 g, 4.75 mmol) in DMF (20 mL) was added 2-nitrobenzyl bromide (5.14 g, 23.8 mmol) and DIEA (7.85 mL, 47.5 mmol). The resultant mixture was stirred at room temperature overnight and then 50 °C for 3 h. The mixture was then concentrated in vacuo and purified by silica gel column chromatography (DCM) to afford **3** (5.06g, 8.31mmol, 175%) as a yellow solid. The crude product could not be completely purified and it can be directly used for the next step.

ESI-MS: $[M+H^+]^+$ calcd for $C_{27}H_{37}N_3O_{11}P^+$ 610.22, found 610.11.



2.3. Synthesis of (4)



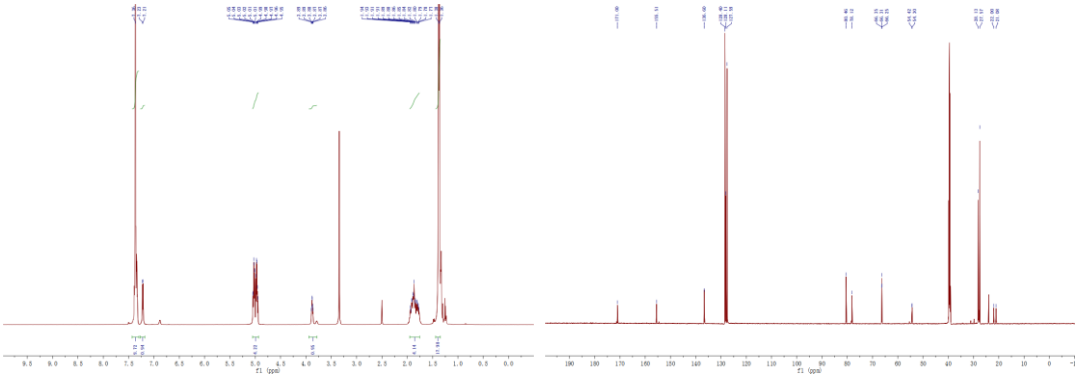
To a stirred mixture of 20 mL trifluoroacetic acid / water / triethylsilane (95 : 2.5 : 2.5) was added **3** (5.06 g, crude product). The mixture was concentrated in vacuo after stirring for 3 h. After the addition of 20 mL water, $NaHCO_3$ was added until the pH reached 8. A solution of Fmoc-OSu (1.77 g, 5.25 mmol) in 1, 4-dioxane (20 mL) was then added and the mixture was stirred overnight. The reaction was concentrated in vacuo and another 30 mL water was added. The aqueous phase was washed with ethyl acetate twice. The resultant solution was treated with 12 N hydrochloric acid until pH reached 2-3. The turbid mixture was extracted with ethyl acetate (6×20 mL). The organic phase was washed with saturated saline (50 mL), dried over anhydrous magnesium sulfate, filtered and concentrated. After purified by silica gel column chromatography (petrol ether/ethyl acetate = 2/1, with 0.5% acetic acid), **4** (2.12g, 3.14mmol) was afforded as faint yellow powder. The total yield of **3** and **4** is 66%.

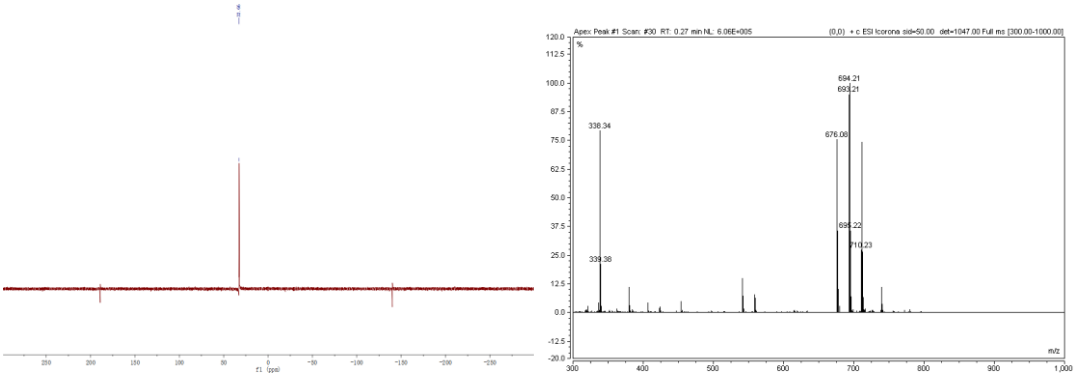
^{1}H NMR (600 MHz, METHANOL-D3) δ 8.00 (dd, J = 8.1, 1.1 Hz, 2H, 3-C**H** in o-NB), 7.70 (d, J = 7.5 Hz, 2H, 4,5-C**H** in Fluorene), 7.64 (s, 2H, 5-C**H** in o-NB), 7.60 (t, J = 7.6 Hz, 4H, 4-C**H** in o-NB and 1,8-C**H** in Fluorene), 7.45 (t, J = 7.4 Hz, 2H, 6-C**H** in o-NB), 7.31 (t, J = 7.5 Hz, 2H, 3,6-C**H** in Fluorene), 7.24 (dt, J = 11.5, 5.7 Hz, 2H, 2,7-C**H** in Fluorene), 5.41 (t, J = 8.4 Hz, 4H, C**H$_2$** in o-NB), 4.37 – 4.21 (m, 3H, C**H** and C**H$_2$** in Fmoc), 4.11 (t, J = 6.8 Hz, 1H, α-C**H**), 2.32 – 2.17 (m, 1H, β-C**H**$_2$), 2.17 – 1.95 (m, 3H, β-C**H**$_2$ and γ- C**H$_2$**).

^{13}C NMR (151 MHz, METHANOL-D3) δ 173.29, 157.30, 147.16, 143.93, 141.27, 133.92, 131.98, 129.10, 128.75, 127.54, 126.92, 124.99, 124.76, 119.69, 66.74, 64.44, 54.02, 24.57, 21.93, 20.98.

^{31}P NMR (243 MHz, METHANOL-D3) δ 34.33.

ESI-MS: $[M+H^+]^+$ calcd for $C_{33}H_{31}N_3O_{11}P^+$ 676.17, found 676.08.





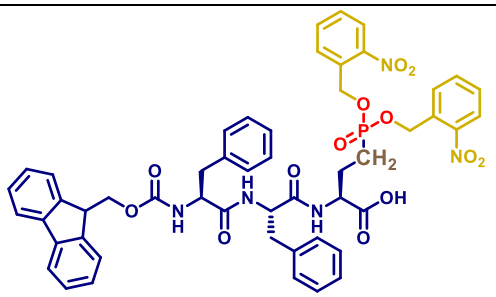
3. Peptide synthesis

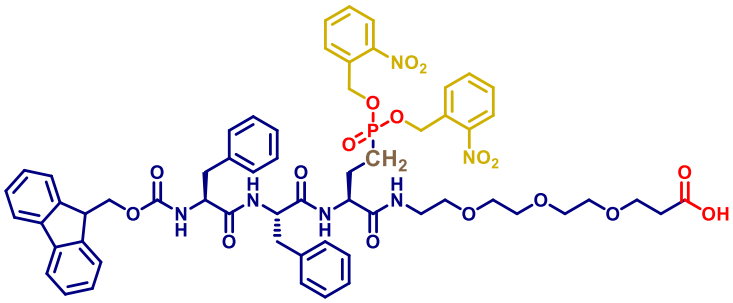
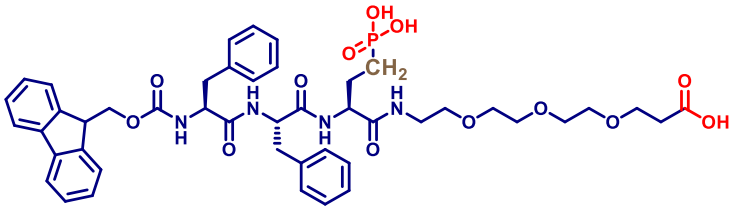
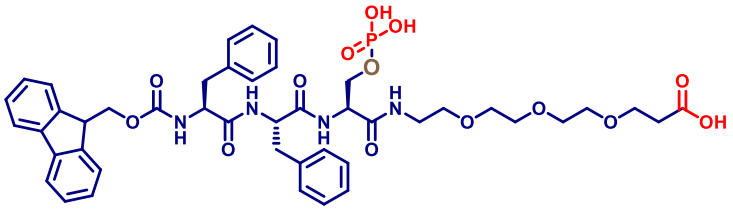
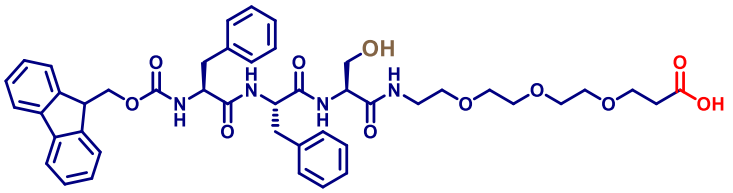
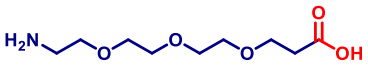
Peptides were synthesized using standard Fmoc solid phase peptide synthesis. The 2-chlorotrityl chloride resin was first swelled in DCM for 30 min. Fmoc-PEG-OH was coupled on the resin at 0.2 mmol/g scale by adding Fmoc-PEG-OH (88.8 mg, 0.2 mmol) and DIEA (8 eq) in DCM to the resin. The following amino acid residues were coupled by adding a mixure of Fmoc-amino acids (4 eq), HATU (3.6 eq), HOAt (4 eq) and DIEA (8 eq) in DMF to the resin. A cocktail mixture composed of TFA : Triisopropylsilane : H_2O in the ratio of 94 : 3 : 3 was added for 2.5 h to cleave peptides from resin. The peptides were puritied using HPLC and molecular weights were confirmed by ESI-MS analysis.

Table S1 Molecular weight of the synthesized peptides.

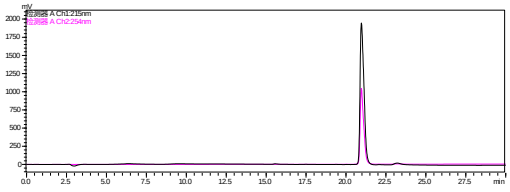
Sequence of peptides	**Theoretical M. W. $[M+H^+]^+$ /g·mol^{-1}**	**Measured M. W. $[M+H^+]^+$ /g·mol^{-1}**
FmocFFpS^C(oNB)-PEG	1173.41	1173.13
FmocFFpS^C-PEG	903.35	903.43
FmocFFpS^O-PEG	905.33	905.41
PEG	222.13	222.18

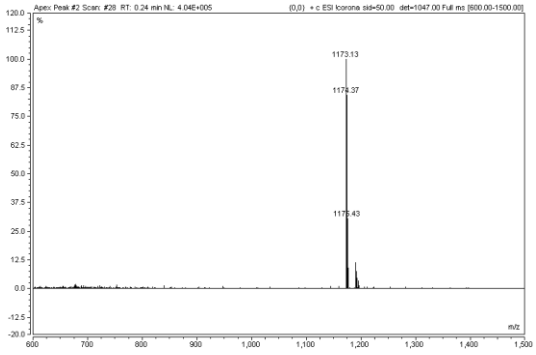
Table S2 Structure of peptides.

Sequence of peptides	**Structure of peptides**
FmocFFpS^C(oNB)	

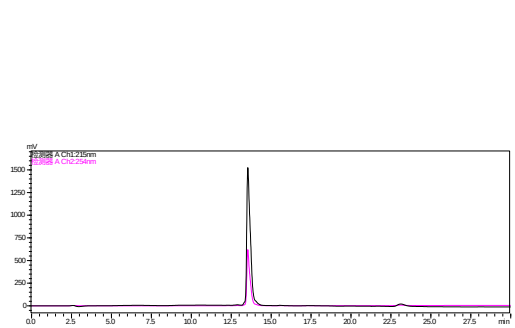
FmocFFpS^{C}(oNB)-PEG	
FmocFFpS^{C}-PEG	
FmocFFpS^{O}-PEG	
FmocFFS-PEG	
PEG	

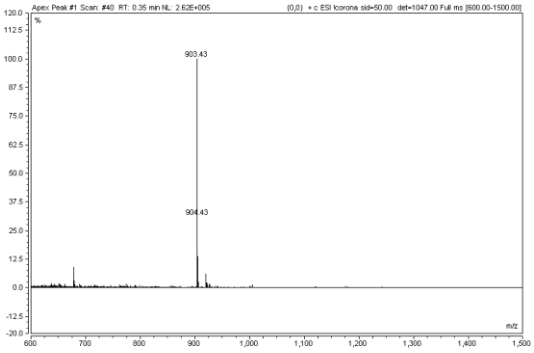
FmocFFpS^{C}(oNB)-PEG, $[M+H^+]^+$ 1173.13, $[M+NH_4^+]^+$ 1190.57



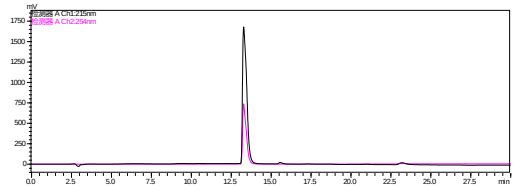


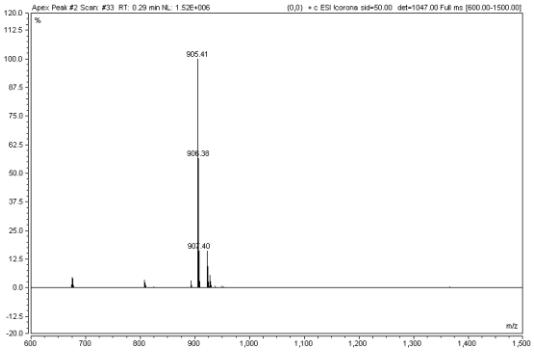
FmocFFpS^{C}-PEG, $[M+H^+]^+$ 903.43, $[M+NH_4^+]^+$ 920.48





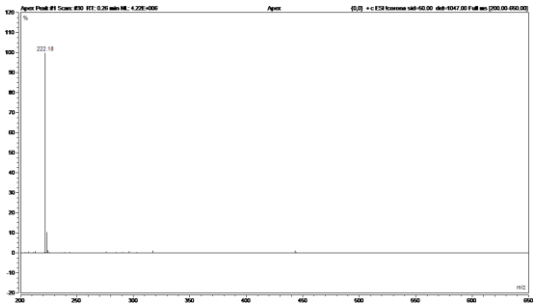
FmocFFpS^{O}-PEG, $[M+H^+]^+$ 905.41, $[M+NH_4^+]^+$ 922.41

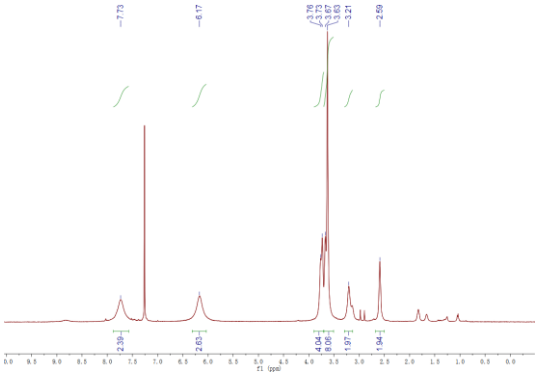




PEG, $[M+H^+]^+$ 222.13, $[M+NH_4^+]^+$ 222.18

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 7.73 (s, 2H), 6.17 (s, 3H), 3.75 (d, J = 12.4 Hz, 4H), 3.65 (d, J = 16.1 Hz, 8H), 3.21 (s, 2H), 2.59 (s, 2H).





4. Secondary structures of the FmocFFpS^{C}(oNB)-PEG assemblies and FmocFFpS^{C}(oNB)-PEG-Ca^{2+} hydrogel (Ca^{2+}-Gel)

4.1. Preparation of peptide solution and gel

20 mg of the lyophilized peptide FmocFFpS^{C}(oNB)-PEG power was added in 10 mL distilled water (dH_2O). 1.0 M NaOH solution was added to the suspension to adjust the pH to ~ 8 – 9. After ultrasounding to form a clear solution, it was adjusted to pH 7.4 by a dropwise addition of 1.0 M HCl. The final concentration of the peptide was 1.70 mM. 3.40 µL of 0.10 M $CaCl_2$ (0.34 µmol) was added into 0.2 mL peptide solution. The peptide solution was allowed to form gel and left undisturbed for 5 h at 37°C, to ensure complete gelation. $CoCl_2$, $NiCl_2$, $CuCl_2$, and $ZnCl_2$ were added by the same method to form different metal-peptide hydrogels.

4.2. Transmission electron microscopy (TEM) measurements

Transmission electron microscope (H-7650B) was provided from Hitachi, Japan. The carbon coated copper grids and uranyl acetate solution were purchased from Beijing Zhongjing Keyi Technology Co., Ltd. The peptide solution or diluted metal-peptide gels was drop casted over the carbon coated copper grids for 3 min. The excess sample was removed by filter paper. 1% w/v uranyl acetate solution was dripped on the copper grids to stain the peptide nanostructures.

4.3. Circular dichroism (CD) measurements

Chirascan-plus Circular Dichroism Spectrometers was provided from Applied Photophysics Ltd, UK. Spectra for all peptides were obtained between a wavelength of 180-260 nm with 1 s signal integrations with a step size of 1 nm and a single acquisition with a slit width of 1 nm. A baseline spectrum for dH_2O was recorded and subtracted from the obtained peptide CD spectra.

5. Zeta potential of assembled FmocFFpS^{C}(oNB)-PEG and Ca^{2+}-Gel

The assembled FmocFFpS^{C}(oNB)-PEG and Ca^{2+}-Gel were diluted at 0.85 mM and the pH was adjusted to 7.4. The samples were measured in the Nano-ZS90 Zetasizer (Malvern Instruments) for 3 times. Zeta-potential was measured to investigate the charge of the assembled peptide and Ca^{2+}-Gel.

Table S3 Zeta Potential of assembled FmocFFpS^{C}(oNB)-PEG and Ca^{2+}-Gel.

	Zeta Potential/mV			
	1	**2**	**3**	**Average**
FmocFFpS^{C}(oNB)-PEG	-57.1	-52.9	-58.6	-56.2
FmocFFpS^{C}(oNB)-PEG + Ca^{2+}	-42.8	-43.4	-43.5	-43.2

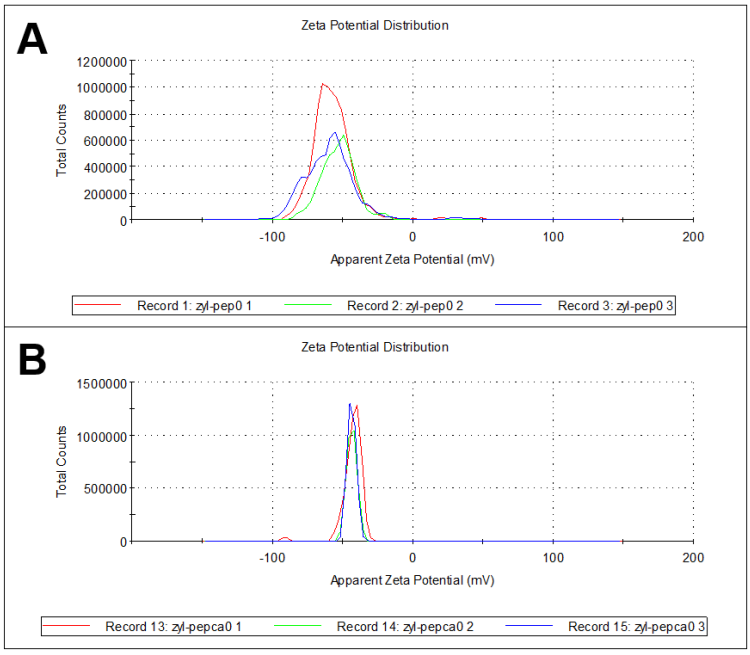


Figure S1. Zeta potential of **(A)** FmocFFpS^{C}(oNB)-PEG, **(B)** FmocFFpS^{C}(oNB)-PEG + Ca^{2+}.

6. Photos of FmocFFpS^{C}(oNB)-PEG with different concentration of Ca^{2+}

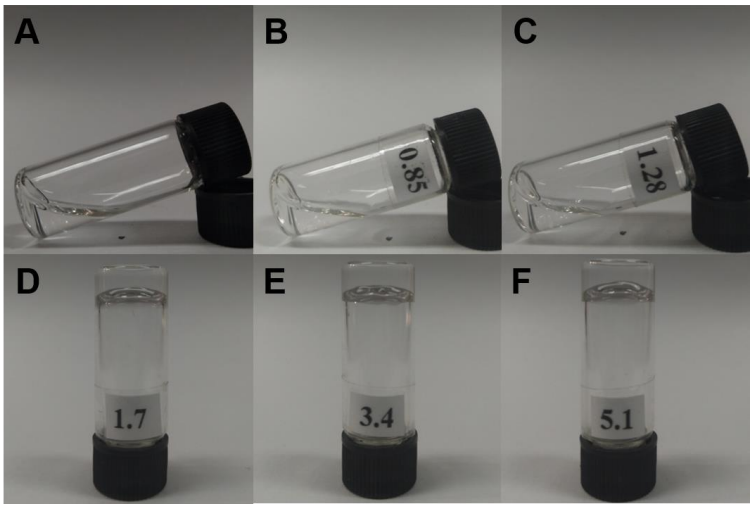


Figure S2. FmocFFpS^{C}(oNB)-PEG was mixed with different concentrations of $CaCl_2$: **(A)** 0 mM $CaCl_2$, **(B)** 0.85 mM $CaCl_2$, **(C)** 1.28 mM $CaCl_2$, **(D)** 1.7 mM $CaCl_2$, **(E)** 3.4 mM $CaCl_2$, **(F)** 5.1 mM $CaCl_2$.

7. FmocFFpS^{C}(oNB)-PEG was diluted by different volume of dH_2O and 1.7 mM Ca^{2+} was added

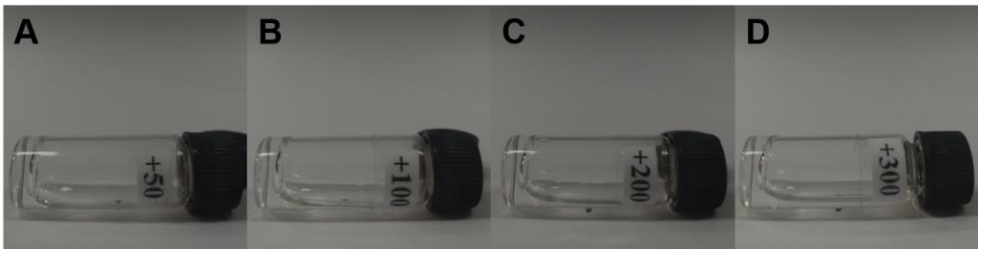


Figure S3. 200 μL 1.7 mM of FmocFFpS^{C}(oNB)-PEG was diluted by different volume of dH_2O, and 1.7 mM Ca^{2+} was added. **(A)** 50 μL dH_2O, **(B)** 100 μL dH_2O, **(C)** 200 μL dH_2O, **(D)** 300 μL dH_2O.

8. FTIR spectra

The FT-IR spectra of peptides were recorded in solid state (KBr) using a Spectrum-100 FTIR spectrophotometer (Perkin Elmer), in the frequency region 450 – 4000 cm^{-1} with a resolution of 1 cm^{-1}. Samples were freeze-dried and grinded with KBr. The background was collected using blank KBr pellet.

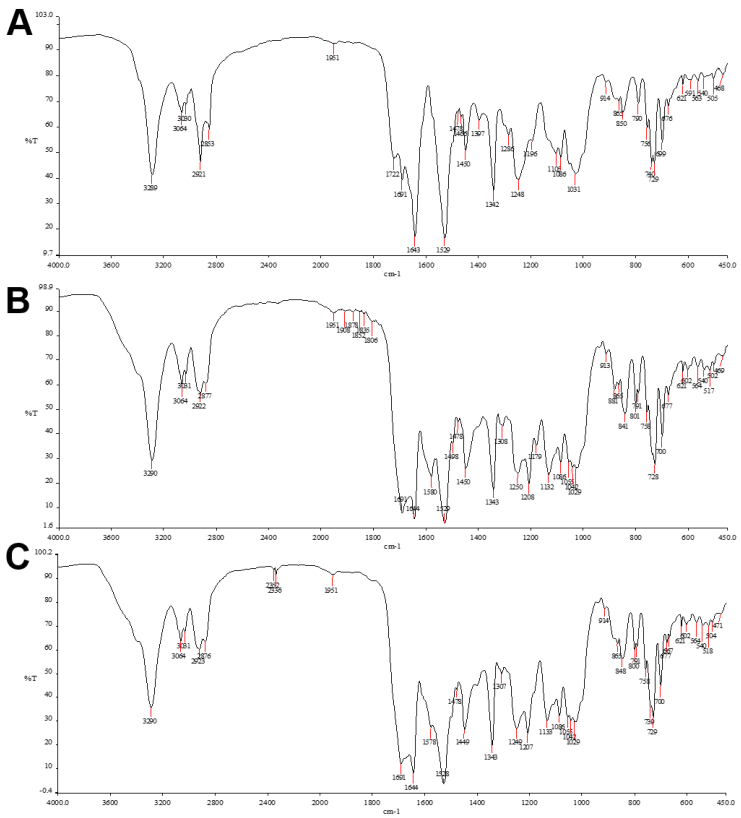


Figure S4. FTIR spectra of **(A)** lyophilized peptide powder, **(B)** lyophilized powder of assembled peptide solution, **(C)** lyophilized powder of Ca^{2+}-Gel.

9. CD and TEM of PEG in the presence of different metal ions

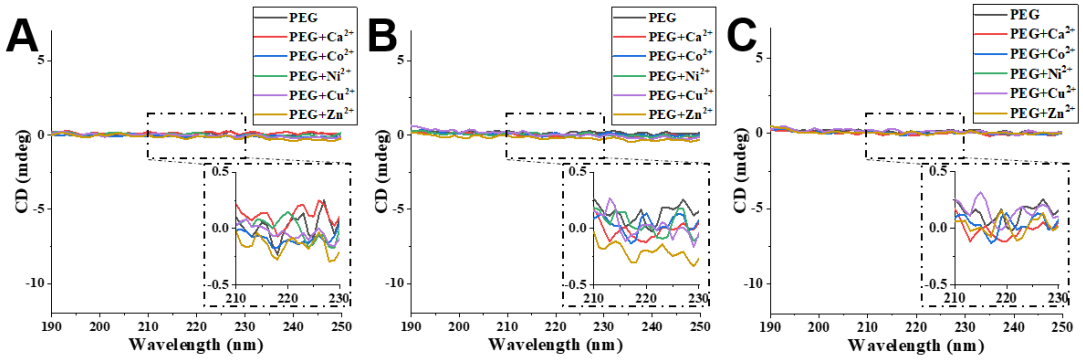


Figure S5 CD spectra of PEG at different concentrations, **(A)** 1.7 mM, **(B)** 17 mM, or **(C)** 40 mM, in the presence of different metal ions.

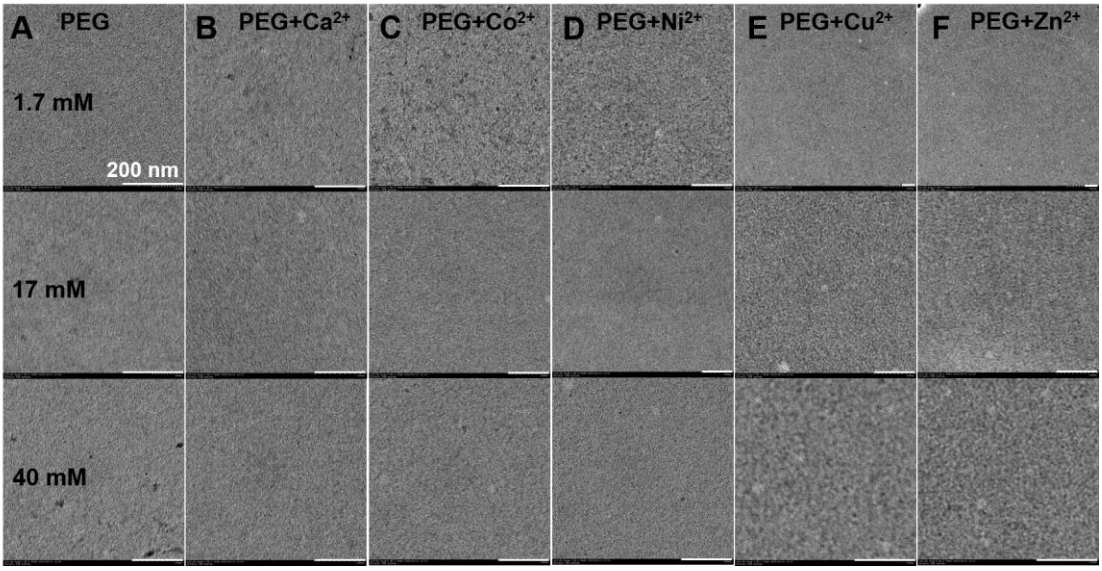


Figure S6 TEM spectra of PEG at different concentrations, **(A)** 1.7 mM, **(B)** 17 mM, or **(C)** 40 mM, in the presence of different metal ions. Scale bar 200 nm.

10. Thermodynamic of FmocFFpS^{C}(oNB)-PEG + Ca^{2+} and FmocFFpS^{C}-PEG + Ca^{2+} asssembling

The calorimetric measurements were conducted using a MicroCal PEAQ-ITC with 200 μL stainless steel sample cell. The sample cell was initially loaded with peptide solution (200 μL, 1.7 mM). The reference cell was loaded with deionized water of the same volume and pH as peptide solution. The calcium chloride solution (40 μL, 17 mM) were continuously injected into the stirred sample cell. The system was stirred at 60 rpm. The observed enthalpies were obtained by integrating the peak areas in the plot of thermal power against time. Then, the calorimetric data were corrected by the mole number of Ca^{2+} per titration to obtain the enthalpy change. The ITC curve were analyzed with standard Marquardt methods in an ITC package. All measurements were conducted at 25.00 ± 0.01 ℃.

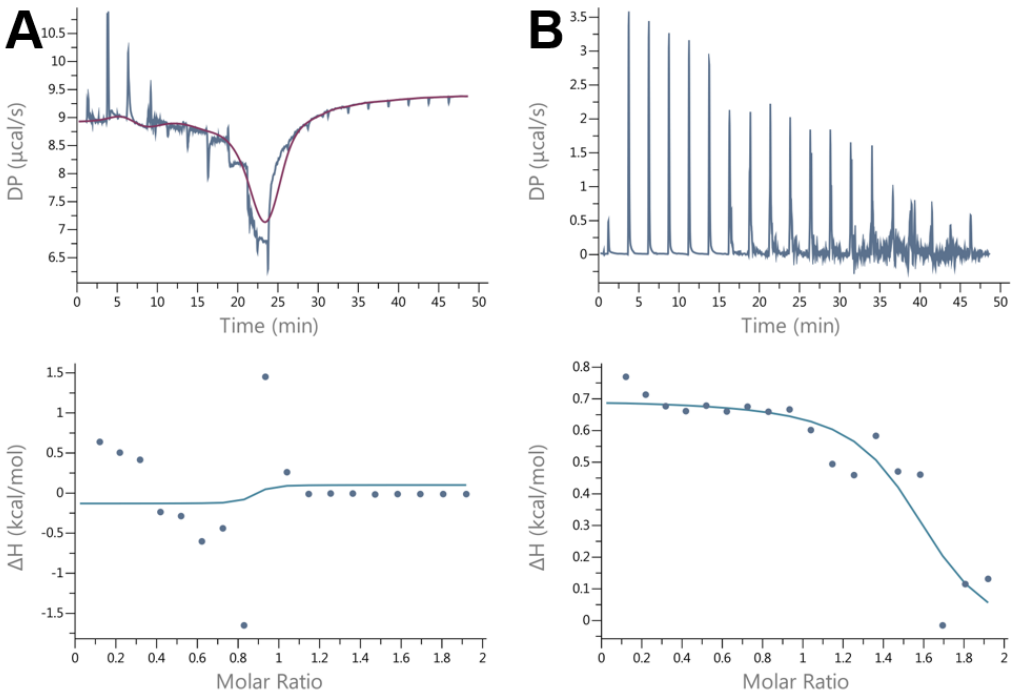


Figure S7 ITC titration curves and enthalpies of (A) FmocFFpS^{C}(oNB)-PEG + Ca^{2+}; (B) FmocFFpS^{C}-PEG + Ca^{2+}.

Table S4 Thermodynamics data of FmocFFpS^{C}-PEG + Ca^{2+}

	ΔH (kcal/mol)	ΔG (kcal/mol)	TΔS (kcal/mol)
FmocFFpS^{C}-PEG + Ca^{2+}	0.764	-6.13	-6.9

11. Kinetic of FmocFFpS^{C}(oNB)-PEG + Ca^{2+} and FmocFFpS^{C}-PEG + Ca^{2+}

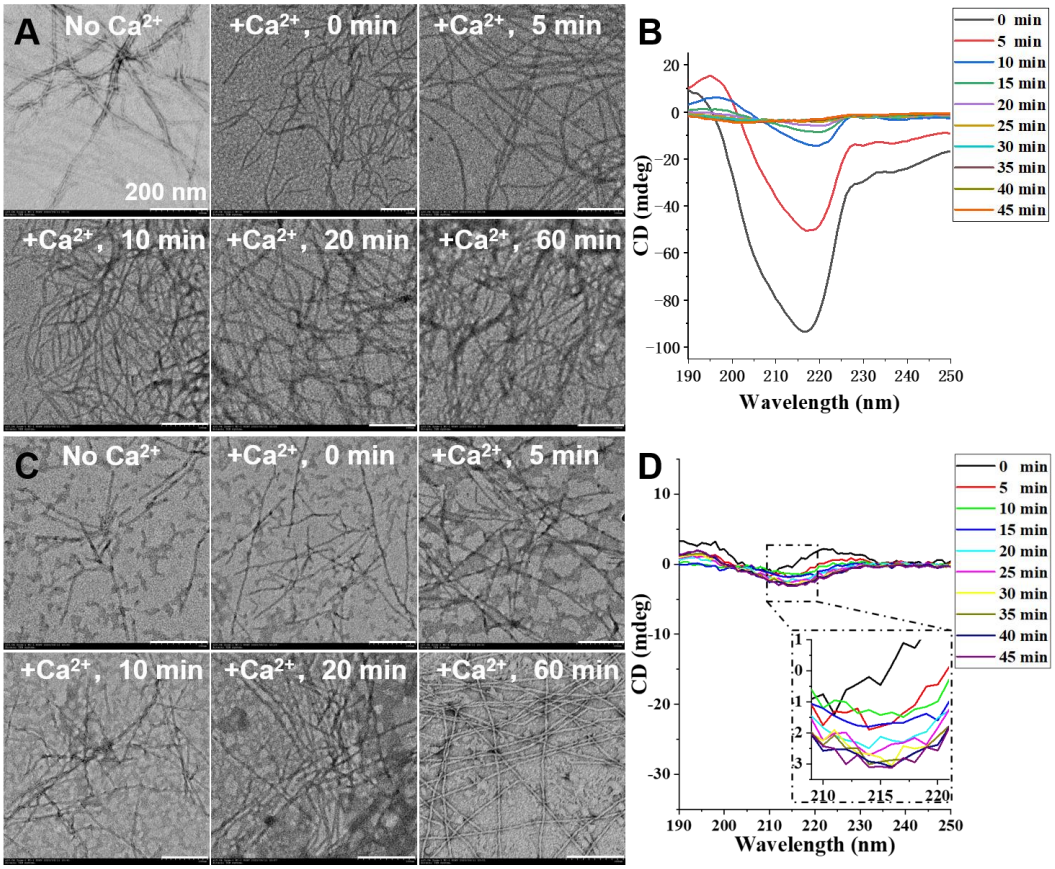


Figure S8 (A) TEM and **(B)** CD spectra of FmocFFpS^{C}(oNB)-PEG + Ca^{2+} over different time; **(C)** TEM and **(D)** CD spectra of FmocFFpS^{C}-PEG + Ca^{2+} over different time. Scale bar 200 nm.

12. Rheological measurements

Rheological studies were performed on an Anton Parr MCR301 rheometer using a 50 mm cone plate geometry. Metal-peptide hydrogels were incubated for 12 h before measurements. The optimum strain values were obtained in the linear viscoelastic region by performing the amplitude sweep. Further the storage modulus (G′) and loss modulus (G″) were recorded as a function of frequency sweep in the range of 0.1 to 100 rad/s. The temperature was controlled at 25 °C.

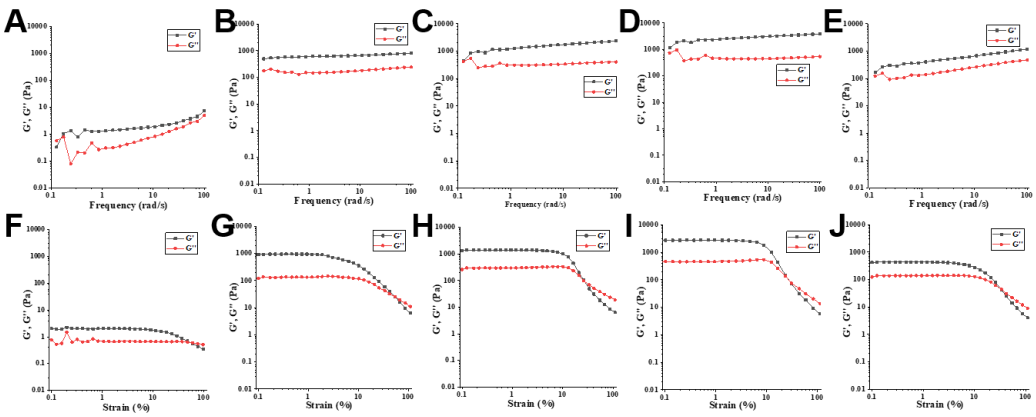


Figure S9. Dynamic frequency sweep of: **(A)** Ca^{2+}-Gel, **(B)** Co^{2+}-Gel, **(C)** Ni^{2+}-Gel, **(D)** Cu^{2+}-Gel, **(E)** Zn^{2+}-Gel;

Dynamic strain sweep of: **(F)** Ca^{2+}-Gel, **(G)** Co^{2+}-Gel, **(H)** Ni^{2+}-Gel, **(I)** Cu^{2+}-Gel, **(J)** Zn^{2+}-Gel.

13. ALP stability of peptides: ALP catalyzed dephosphorylation of peptides

200 μL of 1.70 mM FmocFFpS^{O}-PEG was added by 0.34 μmol $CaCl_2$ and the solution was placed at 37℃ for 10 h. 10 U of alkaline phosphatase was added and the solution was changed to hydrogel within several hours. The hydrogel was analyzed by HPLC and TEM.

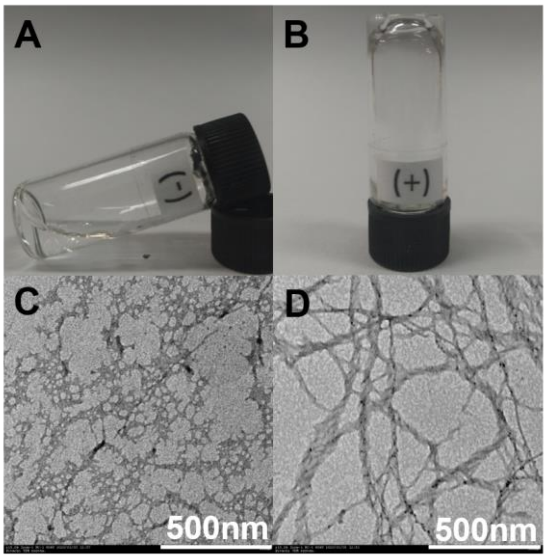
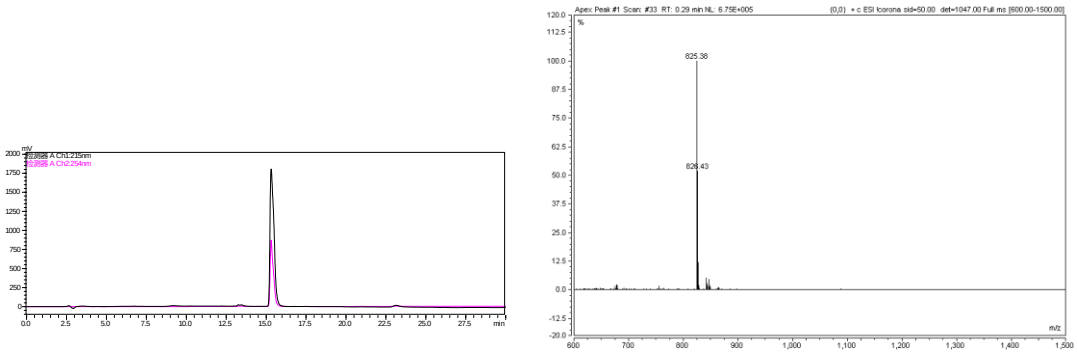


Figure S10. Photos of **(A)** FmocFFpS^{O}-PEG with 1.7 mM $CaCl_2$ without ALP, **(B)** FmocFFpS^{O}-PEG with 1.7 mM $CaCl_2$ and ALP; TEM of: **(C)** FmocFFpS^{O}-PEG with 1.7 mM $CaCl_2$ without ALP, **(D)** FmocFFpS^{O}-PEG with 1.7 mM $CaCl_2$ and ALP.

FmocFFpS^{O}-PEG + Ca^{2+}, (+)ALP, $[M+H^+]^+$ 825.38, $[M+NH_4^+]^+$ 842.49



14. Injectability of peptide gels

200 μL of FmocFFpS^{C}(oNB)-PEG and FmocFFpS^{C}(oNB)-PEG-DOX was injected into 1 mL of 1.70 mM $CaCl_2$ and the solution was placed at 37℃ overnight. Gels formed by inverting of the bottle.

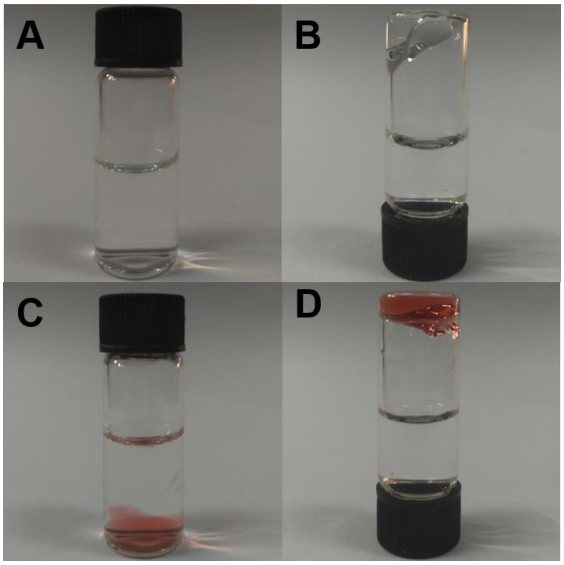


Figure S11. **(A)** and **(B)**: 200 μL of FmocFFpS^{C}(oNB)-PEG injected into 1.70 mM $CaCl_2$; **(C)** and **(D)**: 200 μL of FmocFFpS^{C}(oNB)-PEG-DOX injected into 1.70 mM $CaCl_2$.

15. Release of DOX under UV light or in dark

15.1. UV irradiation experiment

1 mL of peptide solution or Ca^{2+}-Gel was added in 12-well plate and diameter of the well is 22 millimeter. The plate was irradiated by a CEL-M500/350 using 365 nm filter, at a power of 8 mW/cm^2. 100 μL sample was taken out every 30 min. Samples were analyzed by HPLC, TEM and CD.

15.2. DOX release studies from hydrogels

DOX was dissolved at a concentration of 10 mM at first. 500 μL of FmocFFpS^{C}(oNB)-PEG solution was added 10 μL of 10 mM DOX and thoroughly mixed. 0.85 μmol of $CaCl_2$ was added into the solution and the 12-well plate was placed at 37°C for 12 h to form hydrogel. 1.5 mL PBS buffer was added on the gel, and 150 μL of supernatant was replaced by the new PBS buffer every sampling. The plate was placed in dark for 23.5 hours and then under UV for half an hour. The circle was repeated for several times until gels dissolved. Each well was repeated for 4 times in parallel. The negative control was the same as above without UV irradiating and the supernatant was analyzed by UV-visible spectrophotometer.

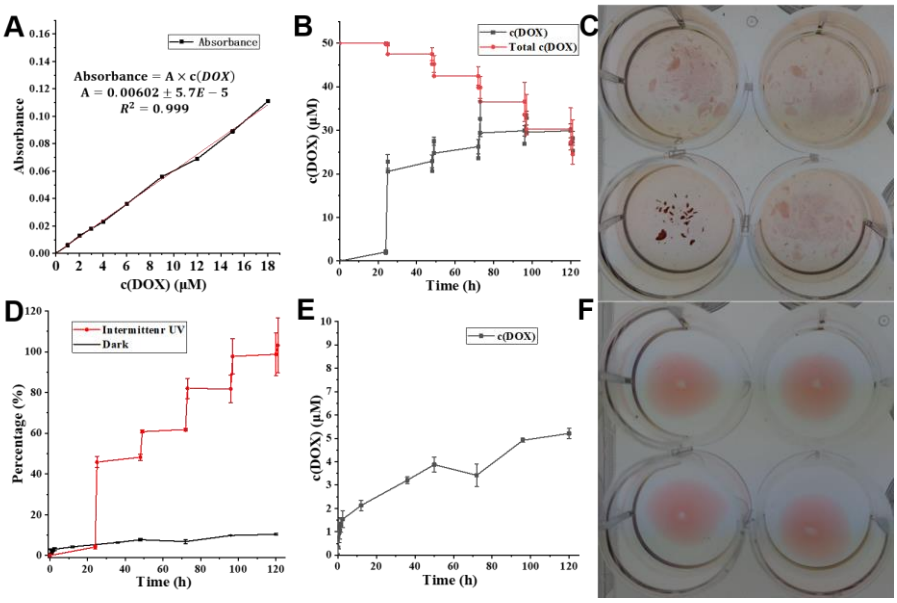


Figure S12. **(A)** Standard curve of absorbance of DOX; **(B)** Remaining total concentration (red) and supernatant concentration (black) of DOX under UV irradiation; **(C)** Photo of Ca^{2+}-Gel-DOX under UV irradiation; **(D)** Supernatant concentration of DOX in dark; **(E)** Release percentage of DOX under UV (red) and in dark (black); **(F)** Photo of Ca^{2+}-Gel-DOX in dark.

16. Stability of DOX under UV light

DOX was dissolved at a concentration of 200 μM. The plate with DOX was placed under UV at a power of 5 mW/cm^2. 100 μL sample was taken out every three quarters. Samples were analyzed by HPLC and MS.

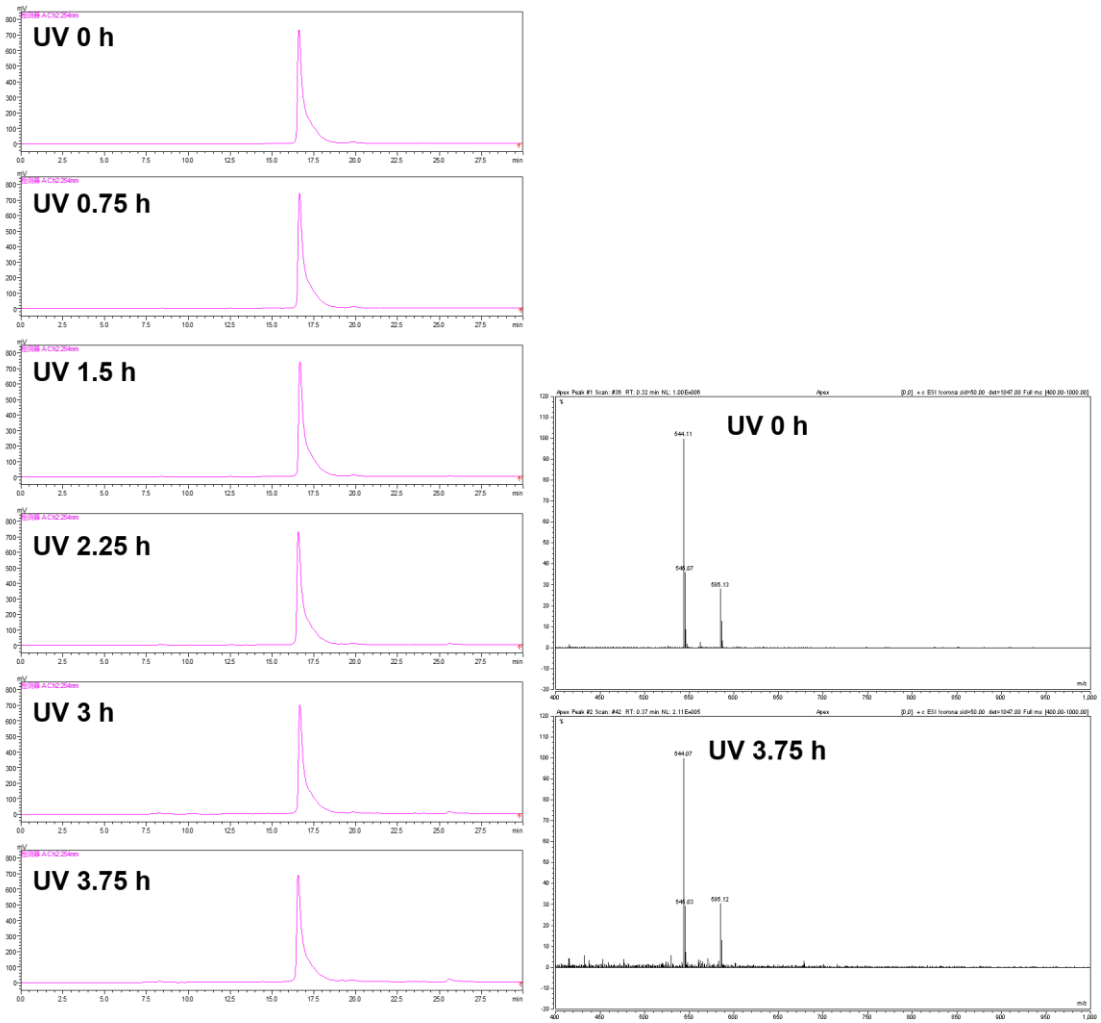


Figure 13 (A) HPLC spectra and **(B)** ESI-MS of DOX before and after UV light irradiation.

References

1. J. Kang, H.-X. Chen, S.-Q. Huang, Y.-L. Zhang, R. Chang, F.-Y. Li, Y.-M. Li and Y.-X. Chen, *Tetrahedron Lett.*, 2017, **58**, 2551-2553.