Rational Design of a Photo-responsive UVR8-derived Protein and a Self-assembling Peptide-Protein Conjugate for Responsive Hydrogel Formation

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Experimental supporting information

Materials and general methods:

Chemicals: Fmoc-amino acids were obtained from GL Biochem (Shanghai, China). Au particles were received from Aladdin. All the other starting materials were obtained from *Alfa*. Chemical reagents and solvents were used as received from commercial sources.

General methods: The synthesized compounds were characterized using ^{1}H NMR (Bruker ARX 400) using DMSO-d_6 as the solvent; HR-MS were received from VG ZAB-HS system (England). HPLC was conducted at LUMTECH HPLC (Germany) system using a C_{18} RP column with MeOH (0.05% of TFA) and water (0.05% of TFA) as the eluents; TEM images were done on a Tecnai G2 F20 system, operating at 200 kV, Rheology was performed on a RheoStress 6000 (HAAKE instrument) system using a parallel plate (20 mm) at the gap of 50 μm. CD spectra were measured using a JASCO J-810 CD spectropolarimeter (Jasco, Japan). With a 0.1-mm path length quartz cell, a wavelength scan was done from 260 to 190 nm at room temperature. All spectra were baseline corrected before analysis.

Protein expression and purification: Expression and purification of protein was using standard recombinant protein technology. Briefly, DNA fragments corresponding to the human TIP-1 were amplified by polymerase chain reaction (PCR). Mutations in TIP-1 were made using a standard PCR-based mutagenesis method and were confirmed by DNA sequencing. To make a single-chain fusion protein of the C-TIP-1, the DNA fragment of C-TIP-1 were cloned into the modified pET32a vector (Novagen). The S-tag and thrombin recognition sites were replaced with a sequence encoding a 3C protease-cleavable segment (Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro). The resulting protein contained a Thioredoxin (Trx)-His6 tag at their N-terminus.

BL21(DE3) CodonPlus *Escherichia coli* cells harboring the expression plasmid were grown in LB medium at 37 °C until the OD_{600} reached 0.6 and then induced with 0.3 mM isopropyl-β-D-thiogalactoside at 16°C for about 16-18 h. After being spun at 5,000 r.p.m. for 15 min, E. coli cells were resuspended in $T_{50}N_{500}I_5$ buffer (50 mM Tris-HCl pH 7.9, 500 mM NaCl and 5 mM imidazole) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1μg/mL leupeptin and 1 μg/mL antipain. The cells were then lysed by sonication. After the lysates had been centrifuged at 18,000 r.p.m. for 30 min, the supernatant was loaded onto a Ni-NTA agarose column (Qiagen) that was equilibrated with $T_{50}N_{500}I_5$ buffer. The Ni-NTA column was washed with 5 column volumes of $T_{50}N_{500}I_5$ buffer. The Trx-his_6-tagged protein was eluted with $T_{50}N_{500}I_5$ buffer

containing 500 mM imidazole. The eluted protein loaded on a HighLoad 26/60 Superdex-200 size-exclusion column (GE Healthcare) and eluted with $T_{50}N_{150}$ buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl) at a flow rate of 2.5 ml/min. Each fraction of the column elute was 5 ml. The protein peak was identified by SDS-PAGE gel. After digestion with PreScission Protease to cleave the N-terminal Trx-His_6-tag, the target protein was purified on a Ni-NTA agarose column (Qiagen) and a HighLoad 26/60 Superdex-200 size-exclusion column. The thiol-maleimide click reaction between the C-TIP-1 and the Nap-GFFYGEK(Maleimide) in the condition of pH=8 (0.1% triethylamine) afford the designed Nap-GFFYGEK(TIP-1). The pure peptide-protein conjugate Nap-GFFYGEK(TIP-1) was obtained by dialysis at 4 ^{0}C for 2 days.

Analytical gel filtration: Size-exclusion chromatography was performed on an AKTA purifier system using a Superdex 200 10/300 column (GE Healthcare) for C-TIP-1 and Nap-GFFYGEK(TIP-1). Protein samples were dissolved in the phosphate buffer saline (PBS, pH 7.4) solutions. The column was calibrated with a gel filtration standard from Bio-Rad.

As can be seen from the figure S-1, these results indicated that these proteins exist in a unique form of oligomers.

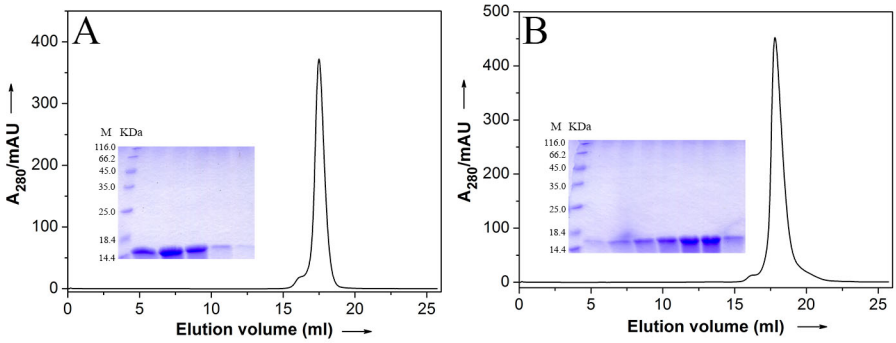


Figure S-1. Analytical gel filtration profiles and their SDS-PAGE gels of different proteins: A) protein of C-TIP-1, B) protein of Nap-GFFYGEK(TIP-1).

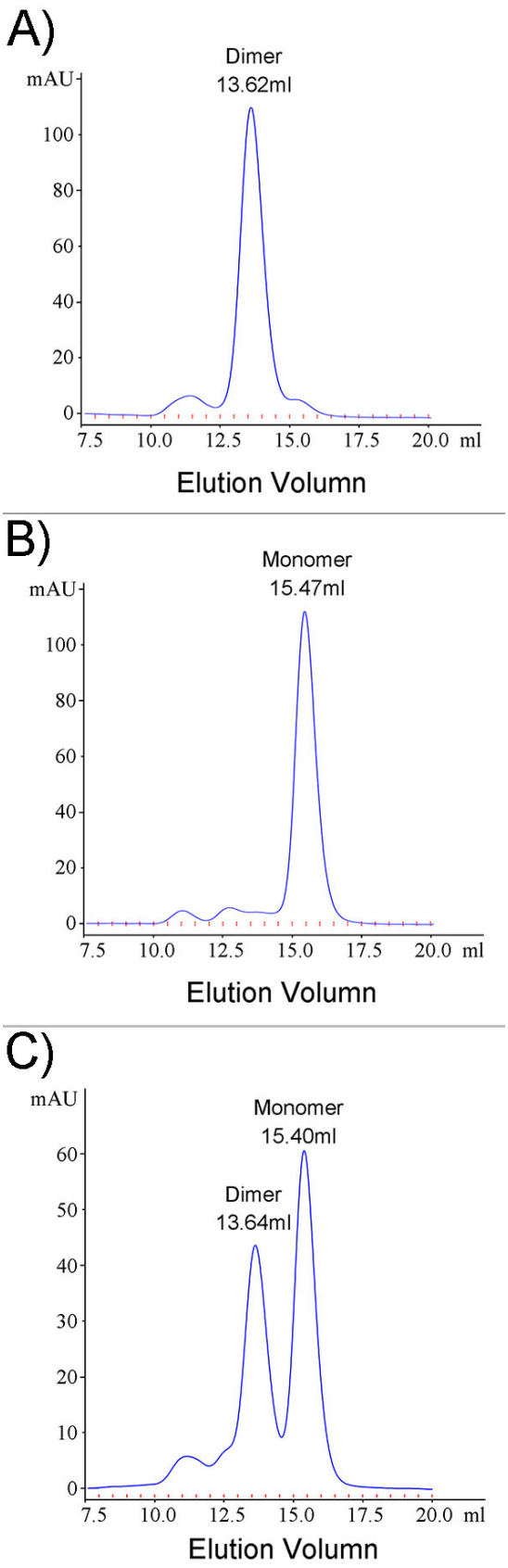


Figure S-2. Analytical gel filtration profiles of the UVR8: A) dimer of UVR8, B) Monomer of UVR8, C) UVR8 of being irradiated by UV light for 20 minutes and then kept in room temperature for 2 hours.

HR-MS of the proteins:

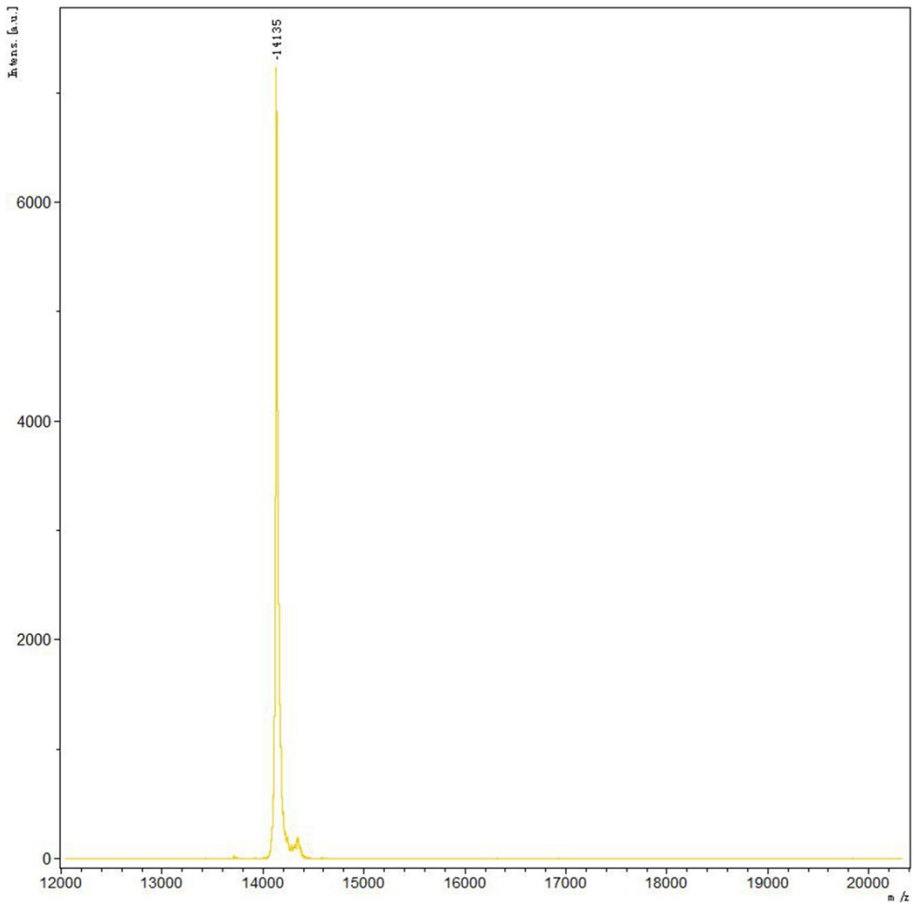


Figure S-3. HR-MS of C-TIP-1.

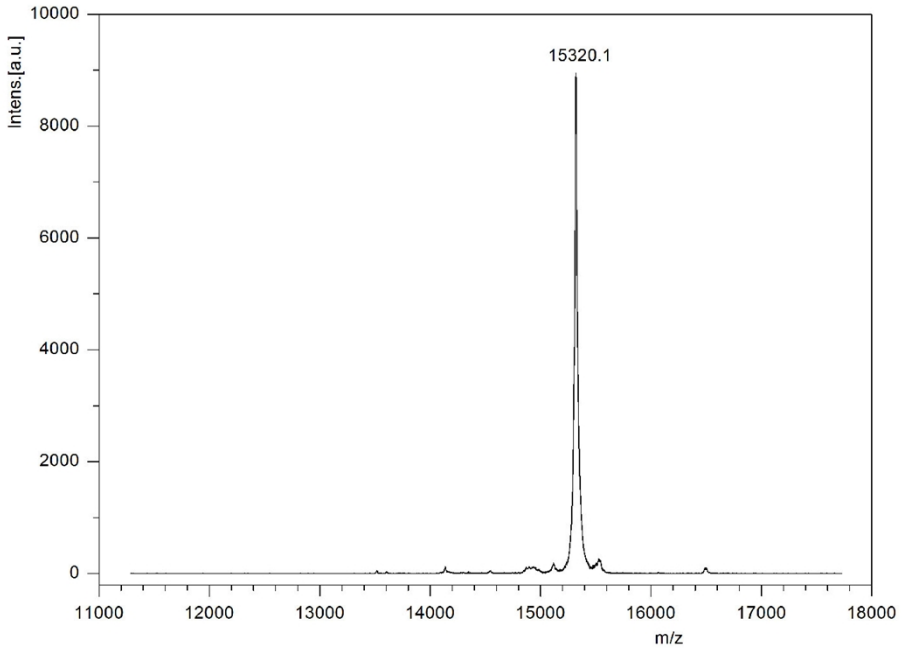


Figure S-4. HR-MS of Nap-GFFYGEK(TIP-1).

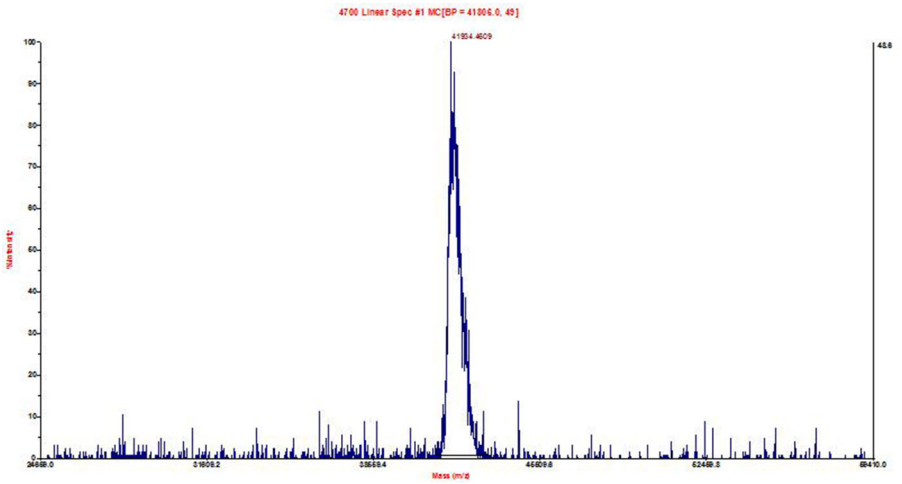


Figure S-5. HR-MS of UVR8-1

Isothermal titration calorimetry (ITC): ITC measurements were carried out on a MicroCal™ Isothermal Titration Calorimeter iTC200 (GE Healthcare) in PBS buffer pH 7.4 at 16 °C. The peptide was dissolved in the same buffer mentioned above and adjusted to pH 7.4. Both peptide and protein solutions were degassed by being spun at 13,000 r.p.m. for 15 min. To measure the binding constants of C-TIP-1 and Nap-GFFYGEK(TIP-1) with the peptide (GGGWRESAI), an initial injection (0.4 μl) followed by 19 injections (2 μl) peptide (0.500 mM) into the calorimeter cell, which was completely filled with protein solution (0.050 mM), were collected at 120s intervals while being stirred at 1000 r.p.m. The titration data and binding plot were analyzed using MicroCal Origin software with one-site binding model.

From the ITC result , the dissociation constant (Kd) of C-TIP-1 with GGGWRESAI peptide was about 33.56±4.92nM and the dissociation number (N) of C-TIP-1 with GGGWRESAI peptide was 1.14. the dissociation constant (Kd) of Nap-GFFYGEK(TIP-1) with GGGWRESAI peptide was about 40.00±2.91nM and the dissociation number (N) of Nap-GFFYGEK(TIP-1) with GGGWRESAI peptide was 0.954.

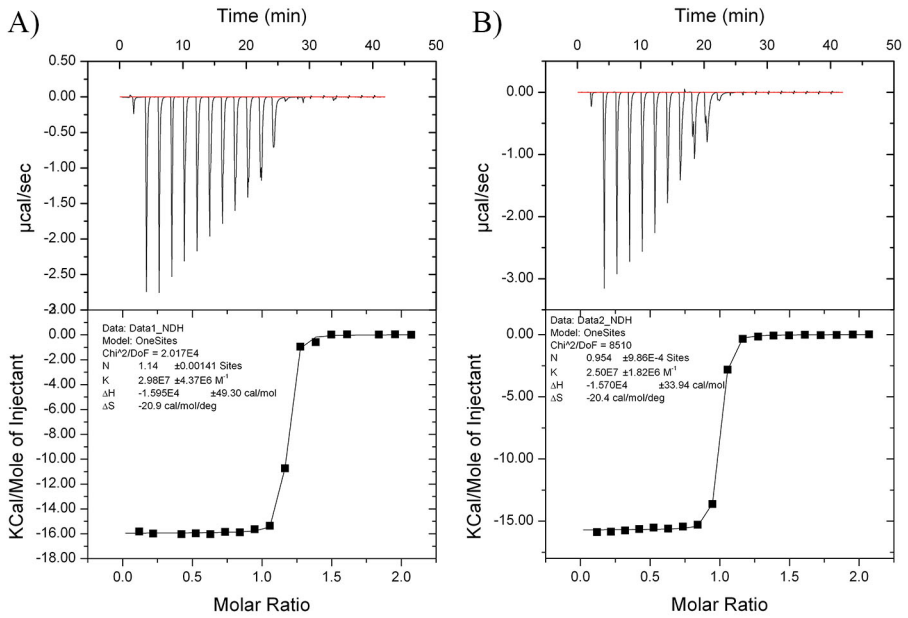


Figure S-6. The dissociation constants (K_d) of proteins with GGGWRESAI peptide were measured by ITC: A) the protein of C-TIP-1; B) the protein of Nap-GFFYGEK(TIP-1).

CD spectra of the proteins:

From the CD result, C-TIP-1 and Nap-GFFYGEK(TIP-1) shared the common feature of α-helix structure in PBS, indicating by the negative peaks near 208 nm and 220 nm. It further proof that the conformation of the conjugate Nap-GFFYGEK(TIP-1) haven't changed by compared with C-TIP-1.

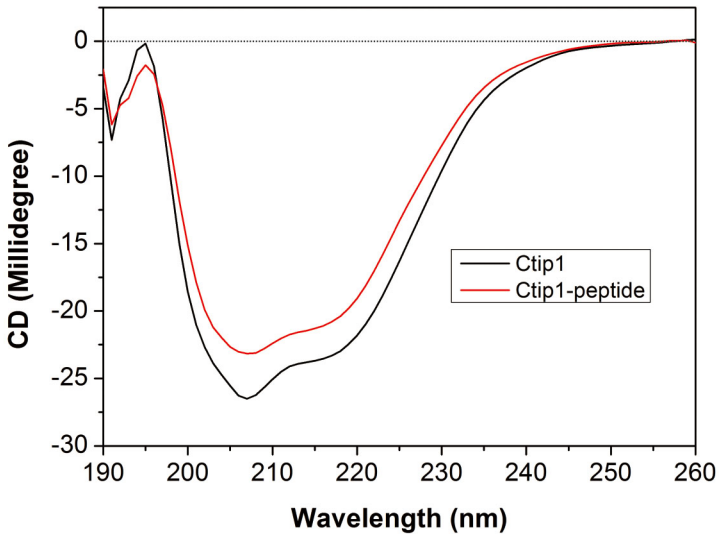


Figure S-7. CD spectra of C-TIP-1 and Nap-GFFYGEK(TIP-1)

DLS:

From the light scattering data, C-TIP-1's diameter is about 4.9nm and Nap-GFFYGEK(TIP-1)'s diameter is about 5.7nm, their diameters are similar. It proved that the morphology of the conjugate Nap-GFFYGEK(TIP-1) haven't changed observably by compared with C-TIP-1.

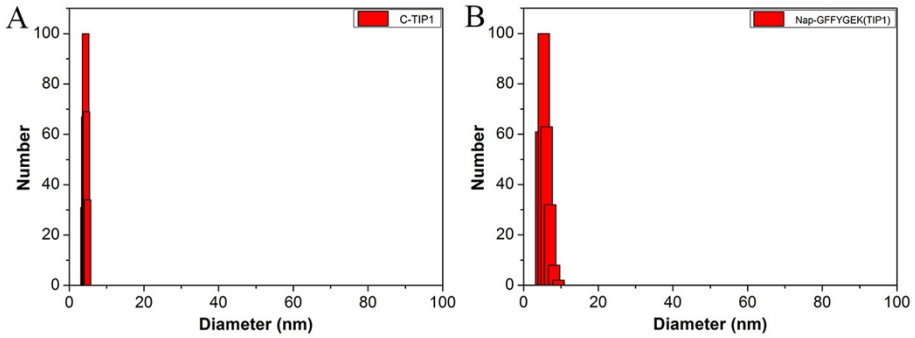
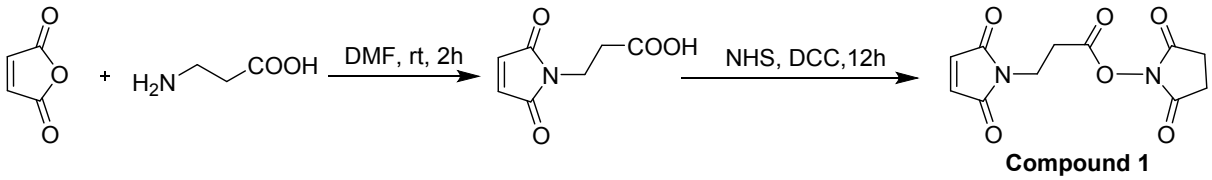


Figure S-8. DLS result of the protein in PBS buffer: A) C-TIP-1 B) Nap-GFFYGEK(TIP-1) .

Synthesis and characterizations:

Peptide systhesis: The peptide derivative was prepared by solid phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin and the corresponding N-Fmoc protected amino acids with side chains properly protected. The first amino acid was loaded on the resin at the C-terminal with the loading efficiency about 0.5 mmol/g. 20% piperidine in anhydrous N,N'-dimethylformamide (DMF) was used during deprotection of Fmoc group. Then the next Fmoc-protected amino acid was coupled to the free amino group using O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU) as the coupling reagent. The growth of the peptide chain was according to the established Fmoc SPPS protocol. At the final step, Naphthalene acetic acid was used to attach on the peptide. After the last coupling step, excessive reagents were removed by a single DMF wash for 5 minutes (5 mL per gram of resin), followed by five steps of washing using DCM for 1 min (5 mL per gram of resin). The peptide derivative was cleaved using 95% of trifluoroacetic acid with 2.5% of TMS and 2.5% of H_2O for 45 minutes. 20 mL per gram of resin of ice-cold diethylether was then added to cleavage reagent. The resulting precipitate was centrifuged for 10 min at 4 ^{0}C at 10,000 rpm. Afterward the supernatant was decanted and the resulting solid was dissolved in DMSO for HPLC separation.

Compound 1 systhesis: β-Alanine (1.35g, 15mmol) was added to a solution of maleic anhydride (1.45g, 15mmol) in 15mL of DMF, then the mixture was stirred for 2 h. The resulting solution was cooled in an ice bath and N-hydroxysuccinimide (2.1g, 18.5mmol) was added followed by DCC(6.45g, 31.5mmol). After 30 min, the ice bath was removed and the solution was vigorously stirred overnight. The white precipitate formed was filtered, washed with DMF (5mL) and the filtrate was poured onto ice. The white precipitate formed in the water was filtered, washed with 10 mL of H_2O and dried in freeze drier to give compound 1 (1.85g, 50%). δ_H (400 MHz, DMSO-d_6) 7.05 (s, 2H), 3.71-3.76 (t, 2H), 3.02-3.07 (t, 2H), 2.79 (s, 4H).



Scheme S-1. Synthetic route for compound 1

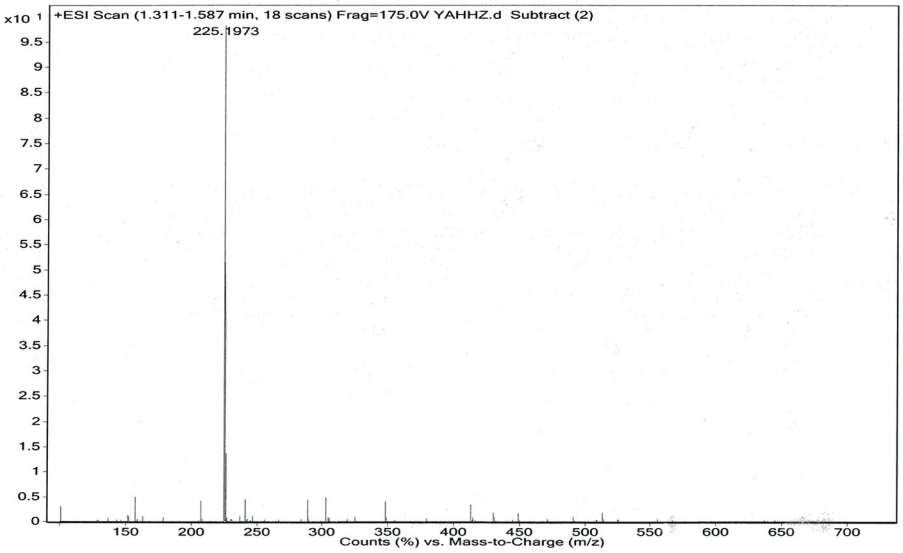
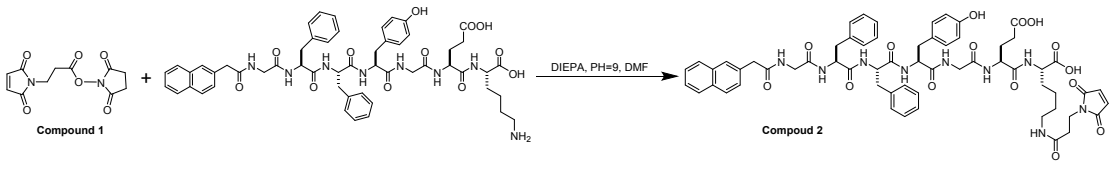


Figure S-9. HR-MS of compound 1

Compound 2 systhesis: we firstly prepared the peptide Nap-GFFYGEK by standard Fmoc- solid phase peptide synthesis (SPPS), then compound 1 (52mg, 0.2mmol) was added to a solution of Nap-GFFYGEK (200mg, 0.2mmol) in 4mL of DMF, diisopropylethylamine (DIEA) (165μL, 1mmol) was added following. the mixture solution was stirred overnight at the condition of pH=9. Then the mixture solution was purified by HPLC. The compound 2 Nap-GFFYGEK(Maleimide) obtained about 150mg (65%). δ_H (400 MHz, DMSO-d_6) 8.17-8.23 (m, 2H), 8.06-8.13 (m, 3H), 7.98-8.02 (d, J=8.35Hz, 1H), 7.80-7.94 (m, 5H), 7.74 (s, 1H), 7.45-7.51 (m, 2H), 7.39-7.44 (m, 1H), 7.11-7.23 (m, 10H), 7.01-7.07 (d, J=8.42Hz, 2H), 6.98 (s, 2H), 6.61-6.67 (d, J=8.43Hz, 2H), 4.43-4.53 (m, 3H), 4.33-4.40 (m, 1H), 4.06-4.13 (m, 1H), 3.72-3.75 (d, J=5.54Hz, 2H), 3.67-3.71 (d, J=5.71Hz, 1H), 3.55-3.63 (m, 6H), 2.99-3.01 (m, 5H), 2.71-2.79 (m, 2H), 2.62-2.69 (m, 1H), 2.24-2.33 (m, 4H), 1.87-1.97 (m, 1H), 1.64-1.80 (m, 2H), 1.57-1.62 (m, 1H), 1.23-1.35 (m, 4H).



Scheme S-2. Synthetic route for compound 2 (Nap-GFFYGEk(Mal)).

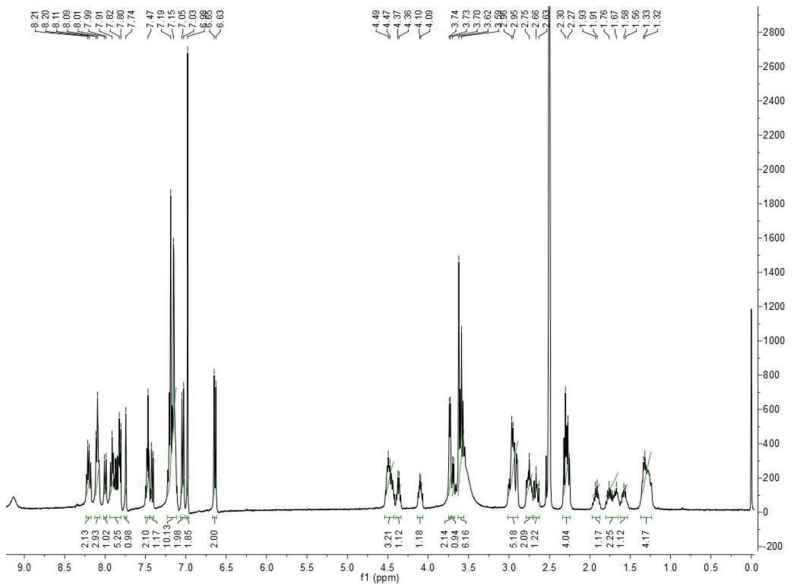


Figure S-10. ^{1}H NMR of compound 2 (Nap-GFFYGEk(Mal)).

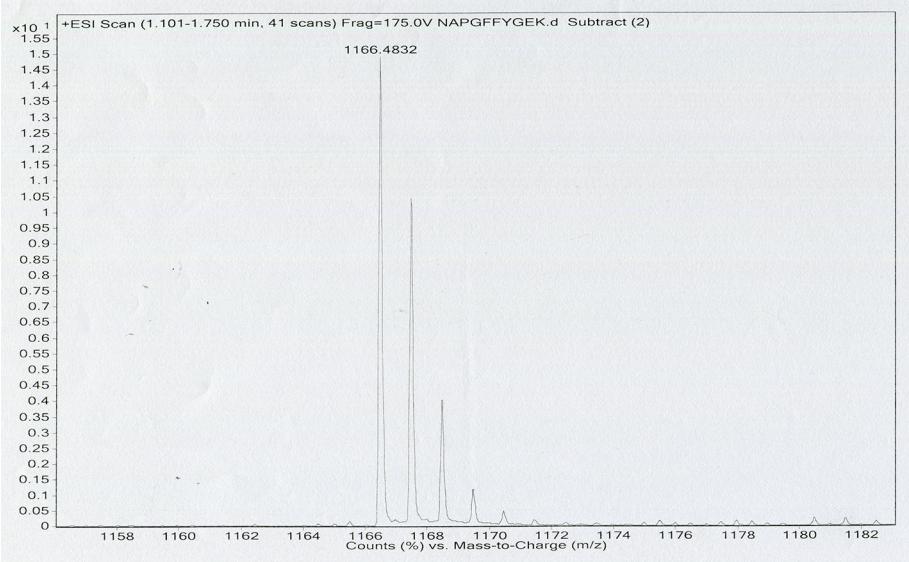
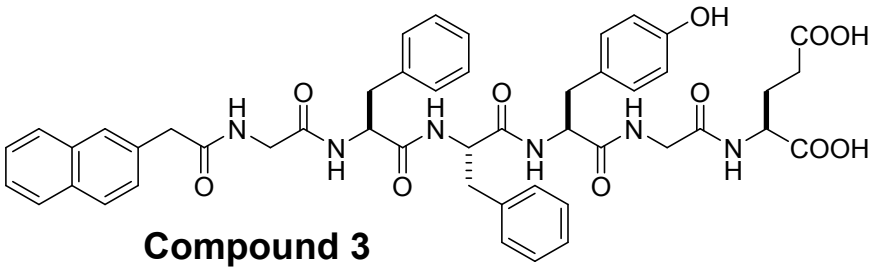


Figure S-11. HR-MS of compound 2 (Nap-GFFYGEk(Mal)).



Scheme S-3. Chemical structure of compound **3** (Nap-GFFYGE)

NapGFFYGE: ^{1}H NMR (300MHz, DMSO-d_6) δ 9.14 (s, 1H), 8.19-8.24 (m, 1H), 8.06-8.15 (m, 3H), 7.99-8.05 (m, 2H), 7.80-7.88 (m, 3H), 7.74 (s, 1H), 7.39-7.51 (m, 3H), 7.12-7.23 (m, 10H), 7.01-7.07 (d, J=8.45Hz, 2H), 6.61-6.67 (d, J=8.44Hz, 2H), 4.41-4.53 (m, 3H), 4.21-4.29 (m, 1H), 3.67-3.76 (m, 3H), 3.54-3.64 (m, 3H), 2.88-3.02 (m, 3H), 2.71-2.79 (m, 2H), 2.62-2.70 (m, 1H), 2.25-2.32 (m, 2H), 1.94-2.03 (m, 1H), 1.75-1.85 (m, 1H).

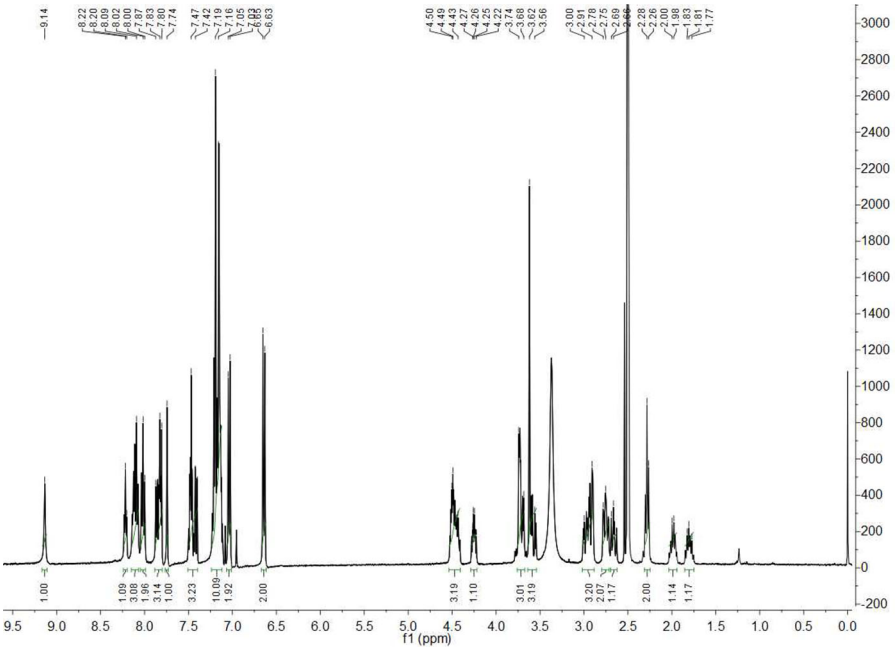


Figure S-12. ^{1}H NMR of compound **3** (Nap-GFFYGE)

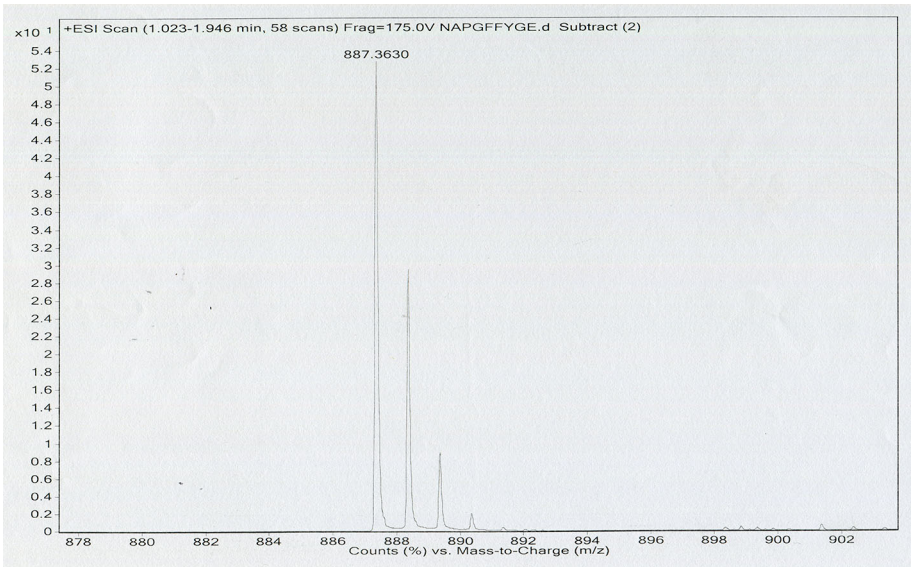
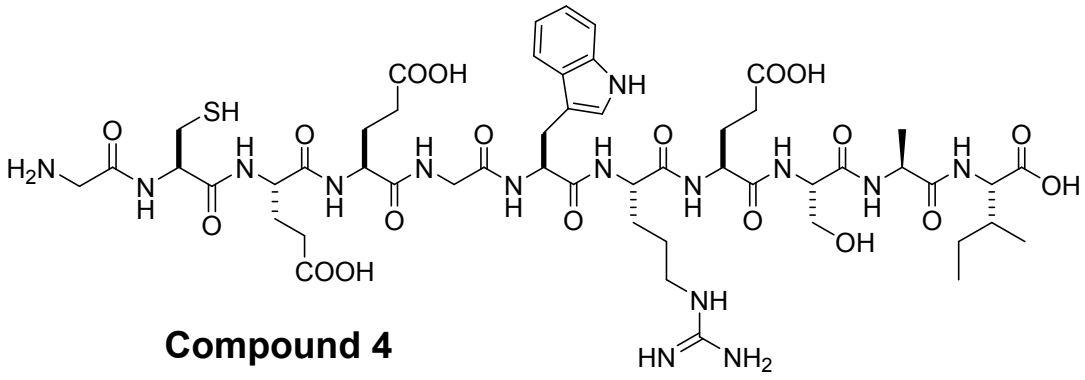


Figure S-13. HR-MS of compound **3** (Nap-GFFYGE)



Scheme S-4. Chemical structure of compound **4** (GCEEGWRESAI)

GCEEGWRESAI: ^{1}H NMR (400MHz, DMSO-d_6) δ 8.63-8.68 (d, 1H), 8.36-8.40 (d, 1H), 8.30-8.35 (m, 1H), 7.92-8.15 (m, 10H), 7.59-7.64 (d, J=7.57Hz, 1H), 7.45-7.51 (m, 1H), 7.29-7.33 (d, 1H), 7.12-7.15 (m, 1H), 7.02-7.07 (t, 1H), 6.95-7.00 (m, 1H), 4.52-4.63 (m, 2H), 4.23-4.40 (m, 6H), 4.12-4.17 (m, 1H), 3.66-3.71 (m, 2H), 3.60-3.64 (m, 2H), 3.54-3.59 (m, 3H), 3.06-3.14 (m, 3H),2.88-2.96 (m, 1H), 2.68-2.79 (m, 2H), 2.21-2.34 (m, 6H), 1.84-1.97 (m, 3H), 1.67-1.81 (m, 5H), 1.38-1.56 (m, 4H), 1.16-1.25 (m, 4H),0.80-0.87 (t, 6H). MS: calc. M^+ = 1235.5, obsvd. $(M+1)^+$ = 1236.5. HR-MS: $(M+1)^+$ = 1236.5294.

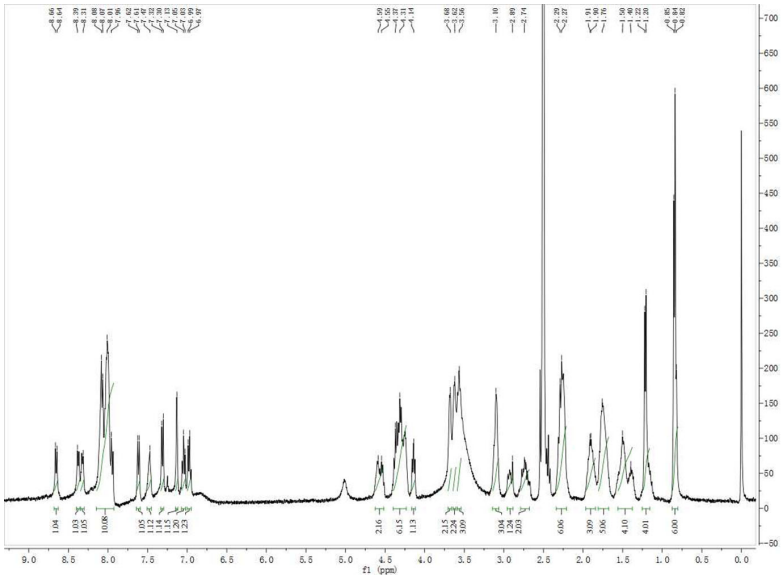


Figure S-14. ^{1}H NMR of compound **4** (GCEEGWRESAI)

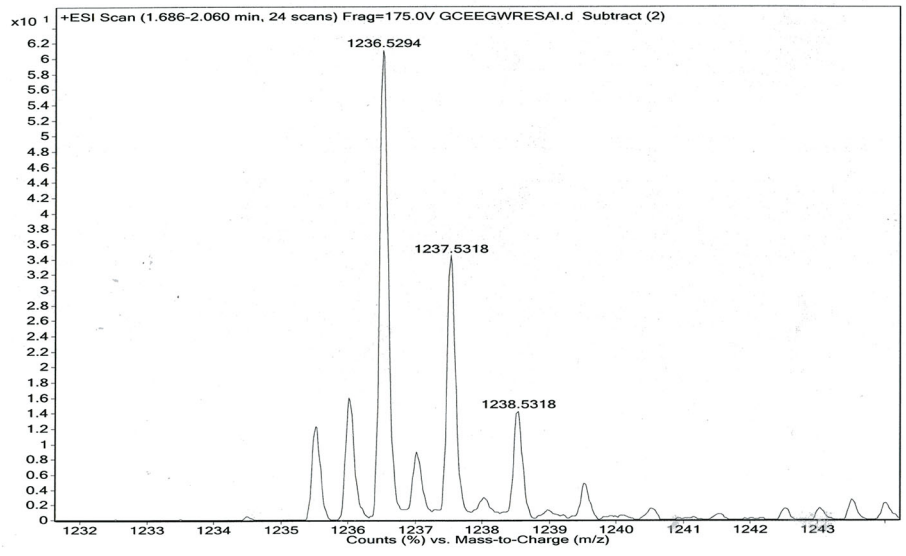
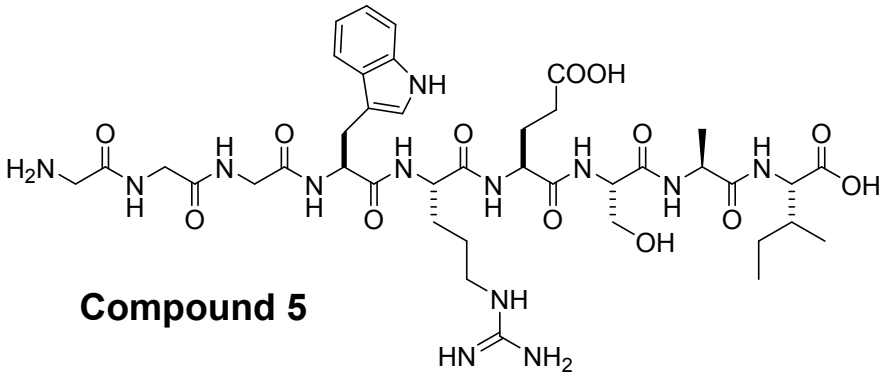


Figure S-15. HR-MS of compound **4** (GCEEGWRESAI).



Scheme S-5. Chemical structure of compound **5** (GGGWRESAI)

GGGWRESAI: ^{1}H NMR (300MHz, DMSO-d_6) δ 8.58-8.66 (t, 1H), 8.27-8.35 (d, J=8.31Hz, 1H), 7.91-8.21 (m, 9H), 7.57-7.64 (t, 1H), 7.27-7.33 (d, J=7.30Hz, 1H), 7.11-7.16 (t, 1H), 6.92-7.07 (m, 2H), 4.52-4.63 (m, 1H), 4.25-4.40 (m, 4H), 4.10-4.16 (m, 1H), 3.71-3.83 (m, 6H), 3.50-3.65 (m, 6H), 3.04-3.17 (m, 3H),2.85-2.97 (m, 1H), 2.23-2.33 (t, 2H), 1.86-1.99 (m, 1H), 1.66-1.84 (m, 3H), 1.34-1.60 (m, 4H), 1.10-1.25 (m, 4H),0.78-0.87 (t, 6H). MS: calc. M^+ = 931.5, obsvd. $(M+1)^+$ = 932.5. HR-MS: $(M+1)^+$ = 932.4584.

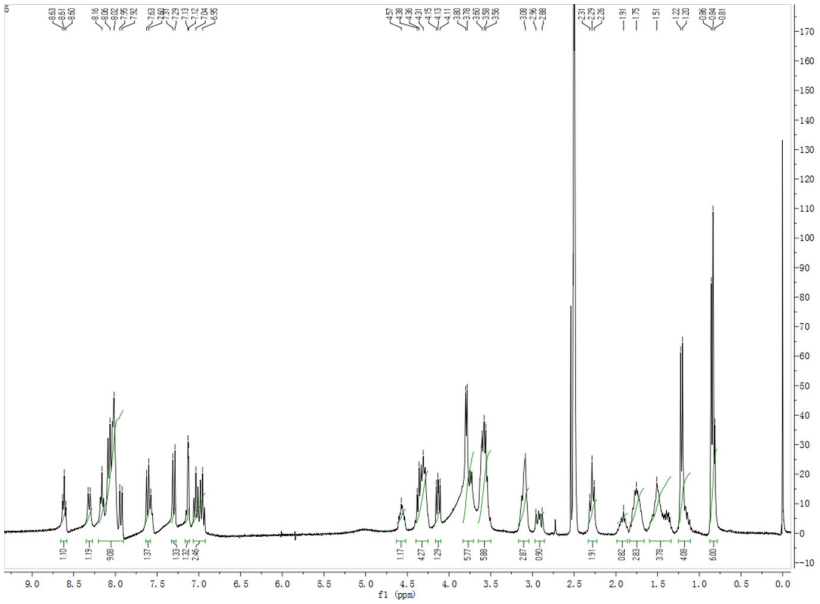


Figure S-16. ^{1}H NMR of compound **5** (GGGWRESAI)

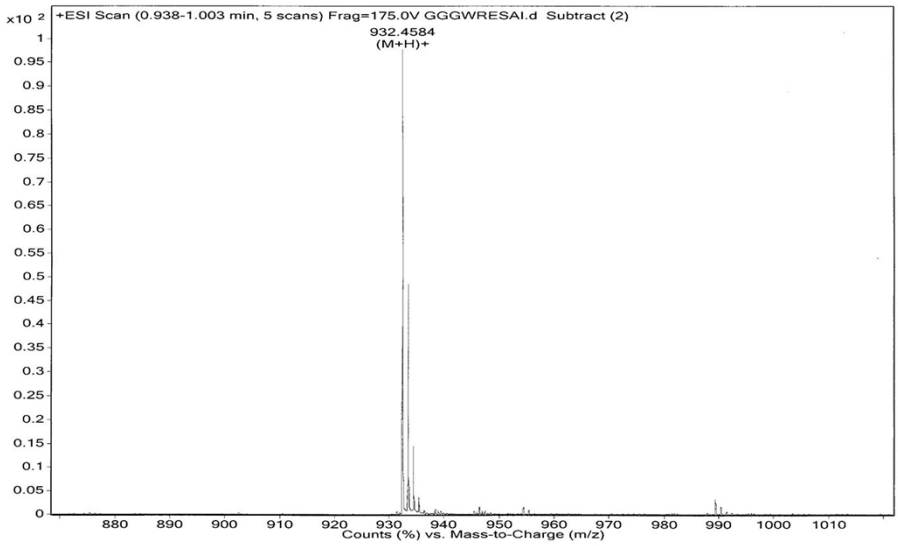


Figure S-17. HR-MS of compound **5** (GGGWRESAI)

Preparation of solution of 3: 2.0 mg of **3** (2.26 μmol) were dissolved in 0.50 mL of PBS buffer solution containing 0.48 mg (2 equiv. to **3**) of Na_2CO_3 (2 equiv. of Na_2CO_3 were used to neutralize the carboxylic acids on **3** to make the final pH value to 7.4). Then heat the solution till it turned into a transparent solution ,cooling to about 40 ^{0}C for use.

Preparation of hydrogel: Take 0.10ml the solution of **3** into a small bottle, and then 0.10 mL of PBS buffer solution containing Nap-GFFYGEK(TIP-1) (0.06mg, 4.0 μmol) and UVR8 (0.17mg, 4.0 μmol, 1 equiv. to Nap-GFFYGEK(TIP-1)) was added. Gels would form after being kept at 7 ^{0}C) for about 1 hour.

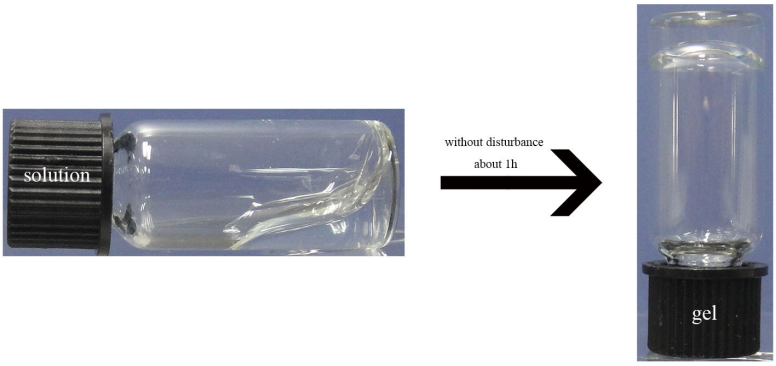


Figure S-18. Optical images of gel formed by treating the solution containing 0.2 wt% of 3, 0.03wt% Nap-GFFYGEK(TIP-1) and 0.168wt% of UVR8-1 (1 equiv. relative to Nap-GFFYGEK(TIP-1)) in PBS buffer solution

Rheology: Rheology test was done on a RheoStress 6000 (HAAKE instrument) system, 20 mm parallel plate was used during the experiment at the gap of 50 μm. The dynamic time sweep was conducted at the frequency of 0.5 rad/s and the strain of 0.2%. Dynamic strain sweep was performed and the strain values within the linear range were chosen for the following dynamic frequency sweep. The gels were characterized by the mode of dynamic frequency sweep in the region of 0.1-100 rad/s at the strain of 0.2%.

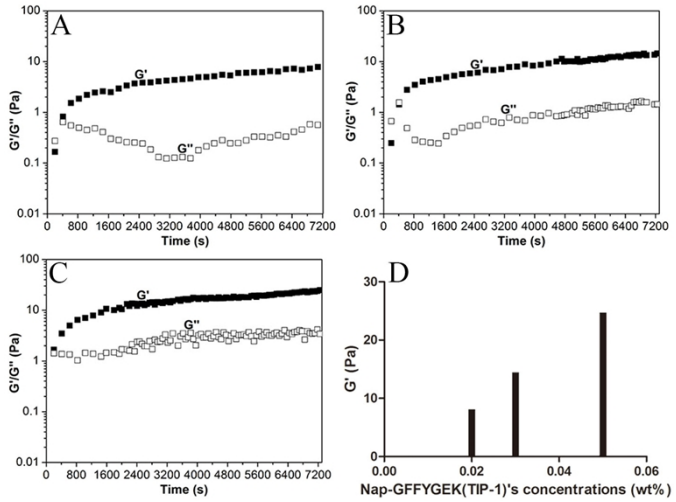


Figure S-19. Rheological measurements with the mode of dynamic time sweep at a frequency of 0.5 rad s^{-1} and strain of 0.2 % for PBS solutions containing 0.2 wt % 3 and different amounts of Nap-GFFYGEK(TIP-1) and UVR8 (1 equiv. relative to Nap-GFFYGEK(TIP-1)) : A) 0.02wt%, B) 0.03wt%, C)0.05wt% and D) The G' of different amounts of Nap-GFFYGEK(TIP-1) and UVR8 at 7200s. (closed symbols: G' and open symbols: G'')

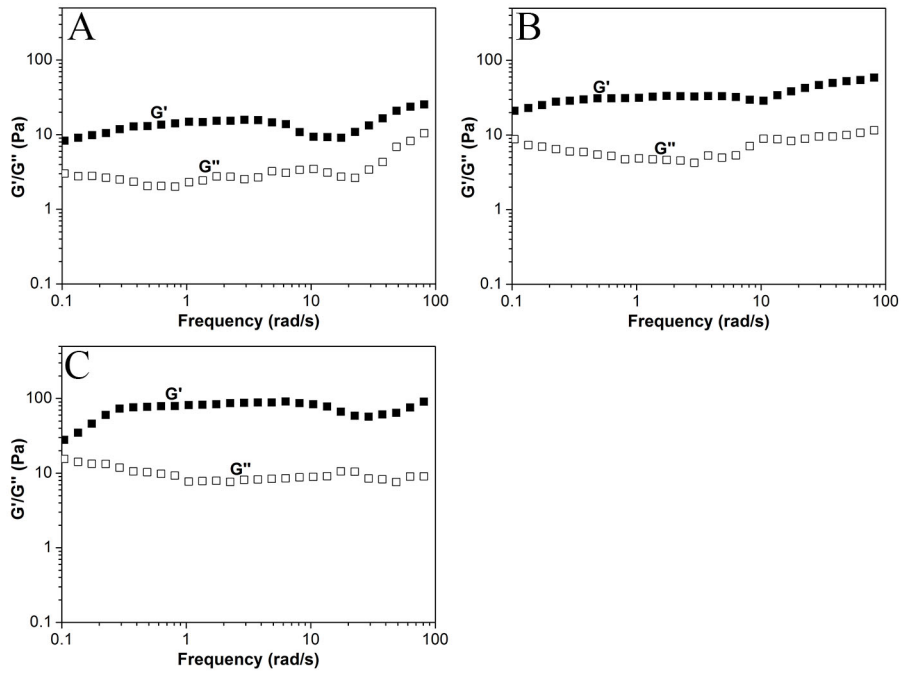


Figure S-20. Rheological measurements with the mode of dynamic frequency sweep at a strain of 0.2 % for PBS solutions containing 0.2 wt % 3 and different amounts of Nap-GFFYGEK(TIP-1) and UVR8(1 equiv. relative to Nap-GFFYGEK(TIP-1)) : A) 0.02wt%, B) 0.03wt% and C)0.05wt%. (closed symbols: G' and open symbols: G'')

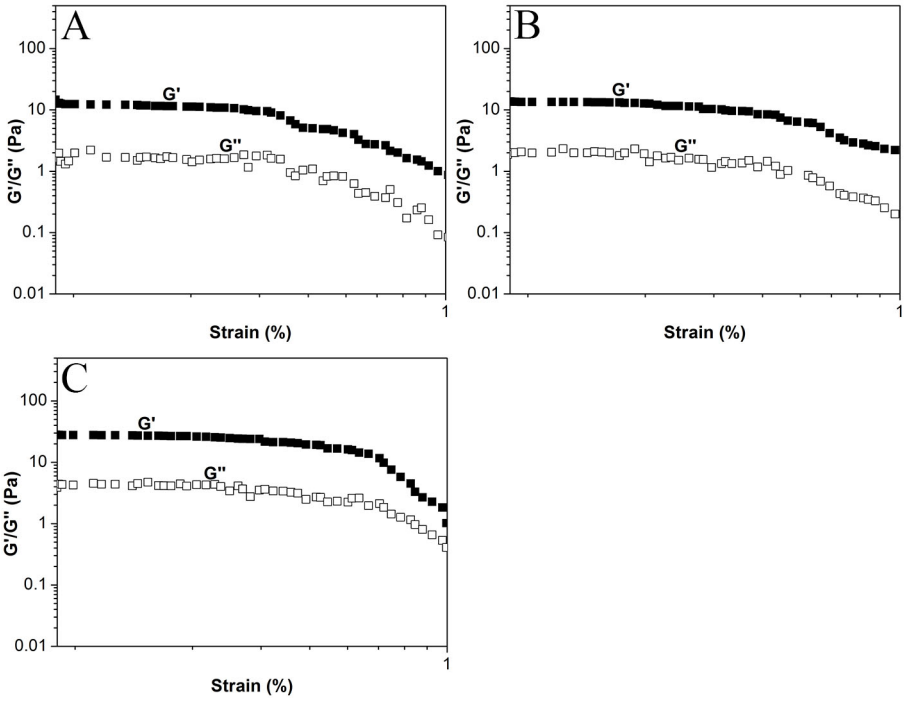


Figure S-21. Rheological measurements with the mode of dynamic Strain sweep at a frequency of 0.5 % for PBS solutions containing 0.2 wt % 3 and different amounts of Nap-GFFYGEK(TIP-1) and UVR8(1 equiv. relative to Nap-GFFYGEK(TIP-1)) : A) 0.02wt%, B) 0.03wt% and

C)0.05wt%. (closed symbols: G’ and open symbols: G’’).

TEM images:

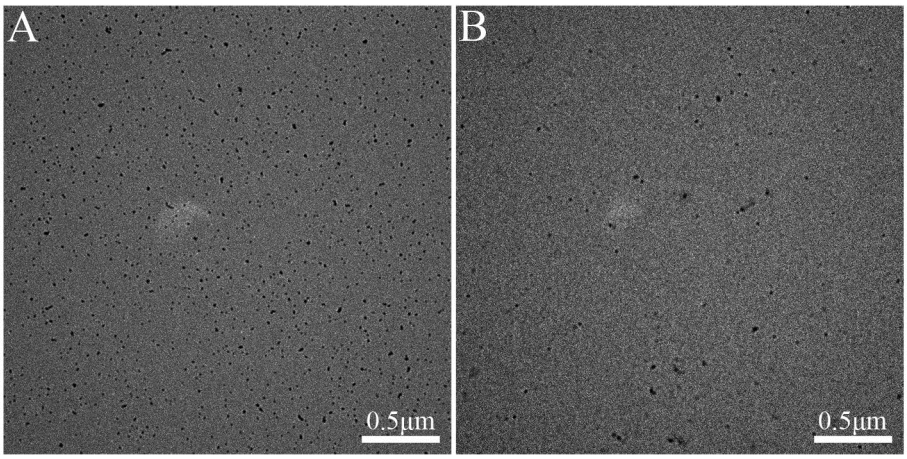


Figure S-22. TEM images: A) bare Au, B) Au modified with peptide of compound 4.

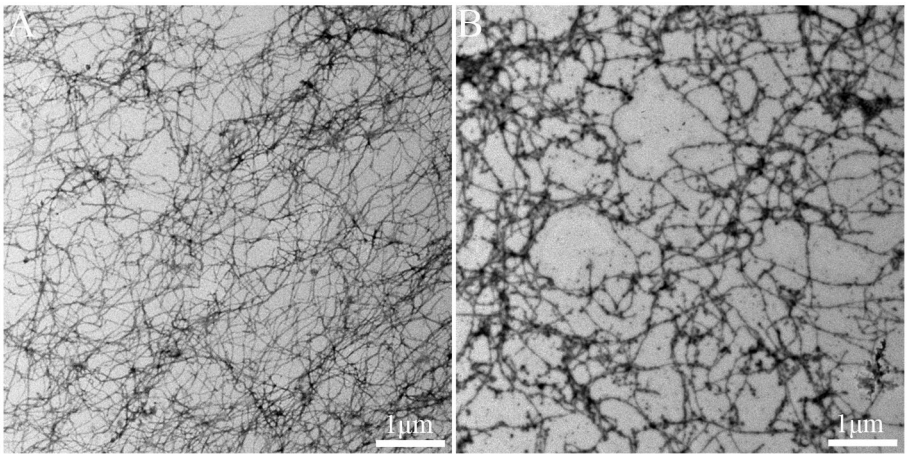


Figure S-23. Compound 3, Nap-GFFYGEK(TIP-1) and A) bare Au, B) Au modified with peptide of compound 4 self-assembled into fibrils that displayed contact epitopes on their surfaces.

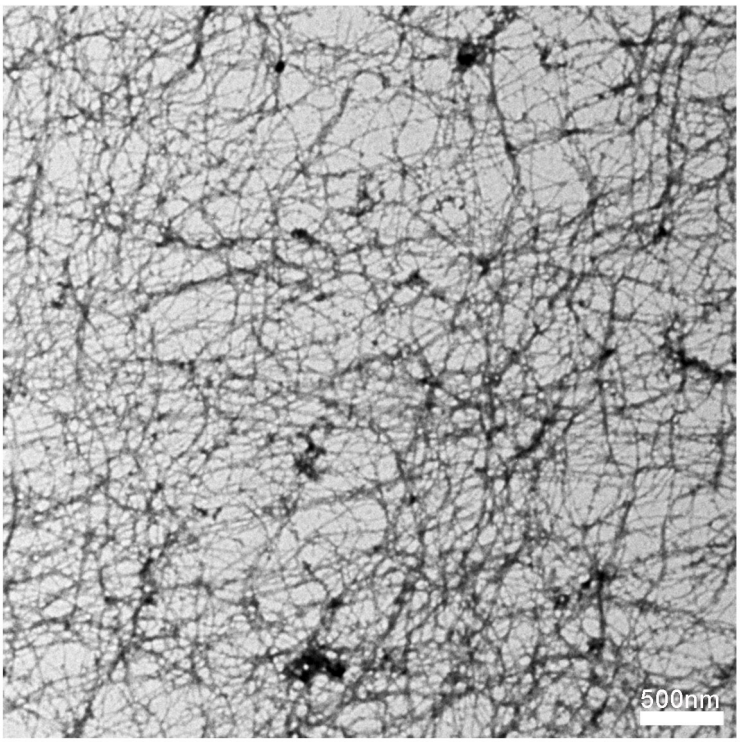


Figure S-24. The TEM image of the UV-irradiated hydrogel

Reversible convertion between gel and solution:

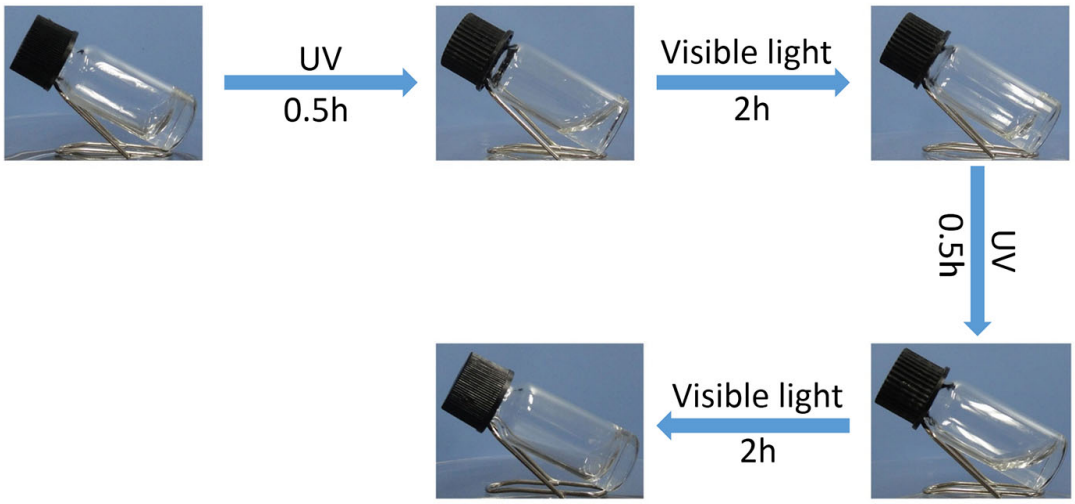


Figure S-25. Optical images to show that the gel and solution are reversible convertion