An enzyme-assisted nanoparticle crosslinking approach to enhance the mechanical strength of peptide-based supramolecular hydrogels

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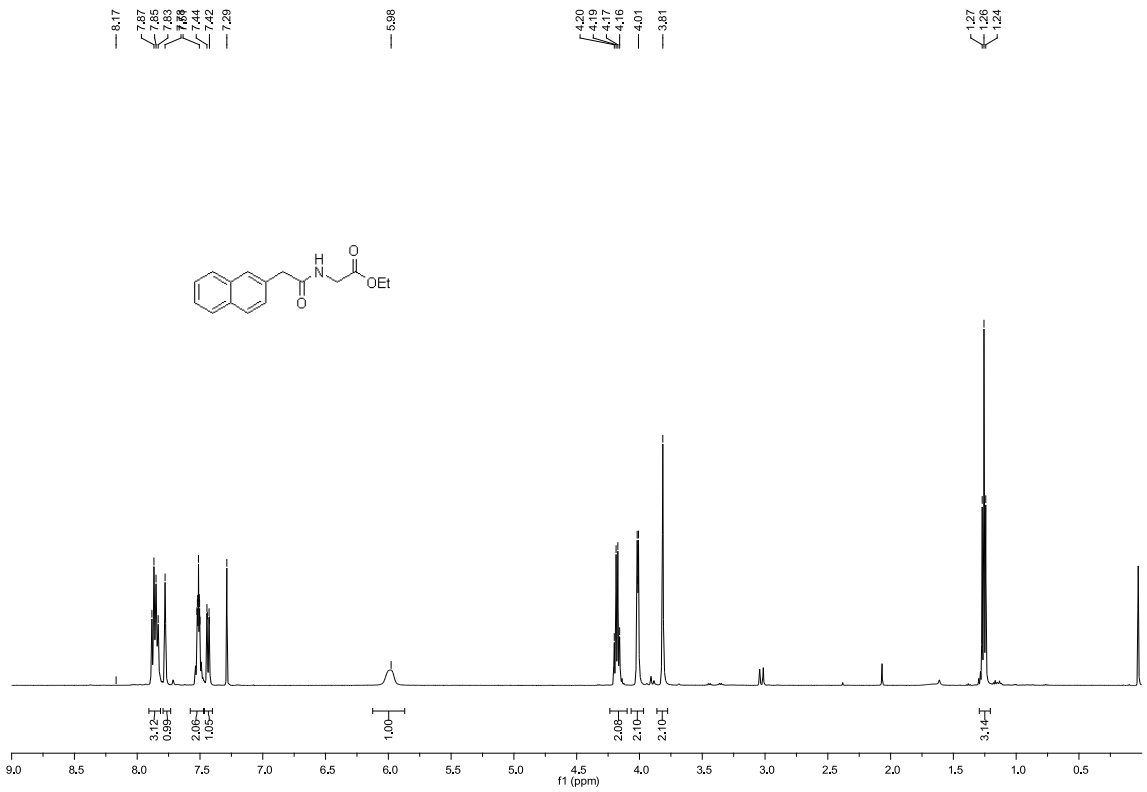
Materials and methods

O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU) was obtained from NovaBioChem. Diisopropylethyl amine (DIPEA) was purchased from Sigma-Aldrich. All other chemicals were obtained from GL Biochem (Shanghai) or Aladdin. All solvents were used directly without further purification.

Synthesis

Synthesis of 2-NapGly-OH

2-Naphthyl acetic acid (1.3 g, 7.0 mmol) was dissolved in DMF (40 mL) with HCl·H-Gly-OEt (1.0 g, 7.16 mmol) and diisopropylethyl amine (DIPEA, 4.5 mL, 25.8 mmol). The clear solution was then added with EDC·HCl (1.4 g, 7.3 mmol) in one portion and the reaction was incubated at room temperature for 12 hr. Then the reaction was poured to 4 N HCl solution (50 mL) and the mixture was extracted with ethyl acetate (EtOAc, 3 × 50 mL). The EtOAc layers were combined, washed with H_2O (2 × 50 mL), saturated Na_2CO_3 solution (1 × 50 mL), H_2O (2 × 50 mL), and brine (1 × 50 mL), and then dried over anhydrous Na_2SO_4. The EtOAc solution was subsequently filtered and concentrated *in vacuo* to give white solid (2-Nap-Gly-OEt)[^{1}H NMR (500 MHz, $CDCl_3$ with TMS as the internal standard, rt): δ(ppm) 1.28 (3 H, t, J= 7Hz), 3.81 (2 H, s), 4.01 (2 H, m), 4.18 (2 H, q, J= 7Hz), 5.98 (1 H, br, s), 7.43 (1 H, d, J= 10 Hz), 7.50 (2 H, m), 7.78 (1 H, s), 7.87 (3 H, m)]. The white product was dissolved in THF (40 mL) and H_2O (20 mL) in an ice-water bath and $LiOH·H_2O$ (1.4 g, 33.3 mmol) was added. The resulting mixture was stirred over the ice-water bath for 2 hr and concentrated *in vacuo* to remove the volatile. The aqueous slurry was then extracted once with CH_2Cl_2 (30 mL) and the aqueous layer (slurry like) was then acidified with 4 N HCl to pH ~1. EtOAc (3 × 50 mL) was added to extract the aqueous layer, combined, washed with H_2O (3 × 50 mL) and brine (1 × 50 mL), and dried over anhydrous Na_2SO_4. The EtOAc solution was filtered and the solvent was removed to provide white solid as the desired product, which was further dried *in vacuo*. Yield: 1.4 g, 85.7% over two steps. ^{1}H NMR (500 MHz, $CDCl_3$ with TMS as the internal standard, rt): δ(ppm) 3.75 (2 H, s), 3.93 (2 H, s), 7.40 (1 H, m), 7.46(2 H, m), 7.73 (1 H, m), 7.80 (3 H, m).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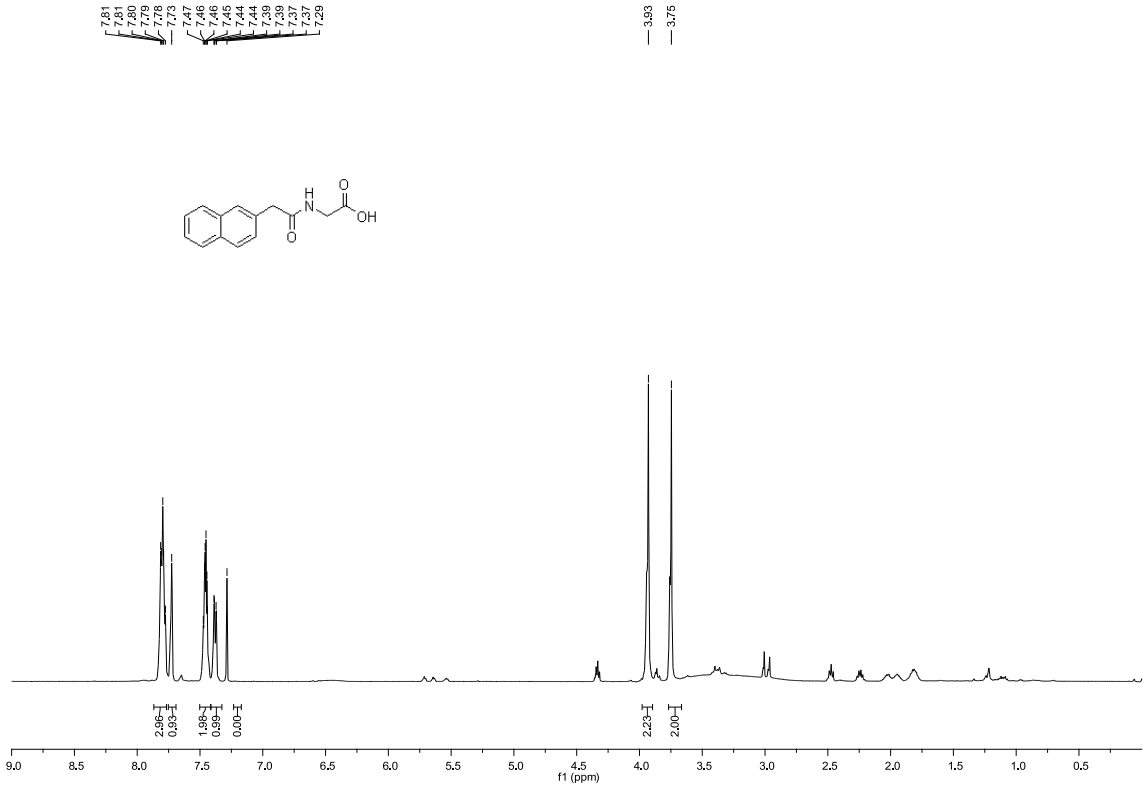
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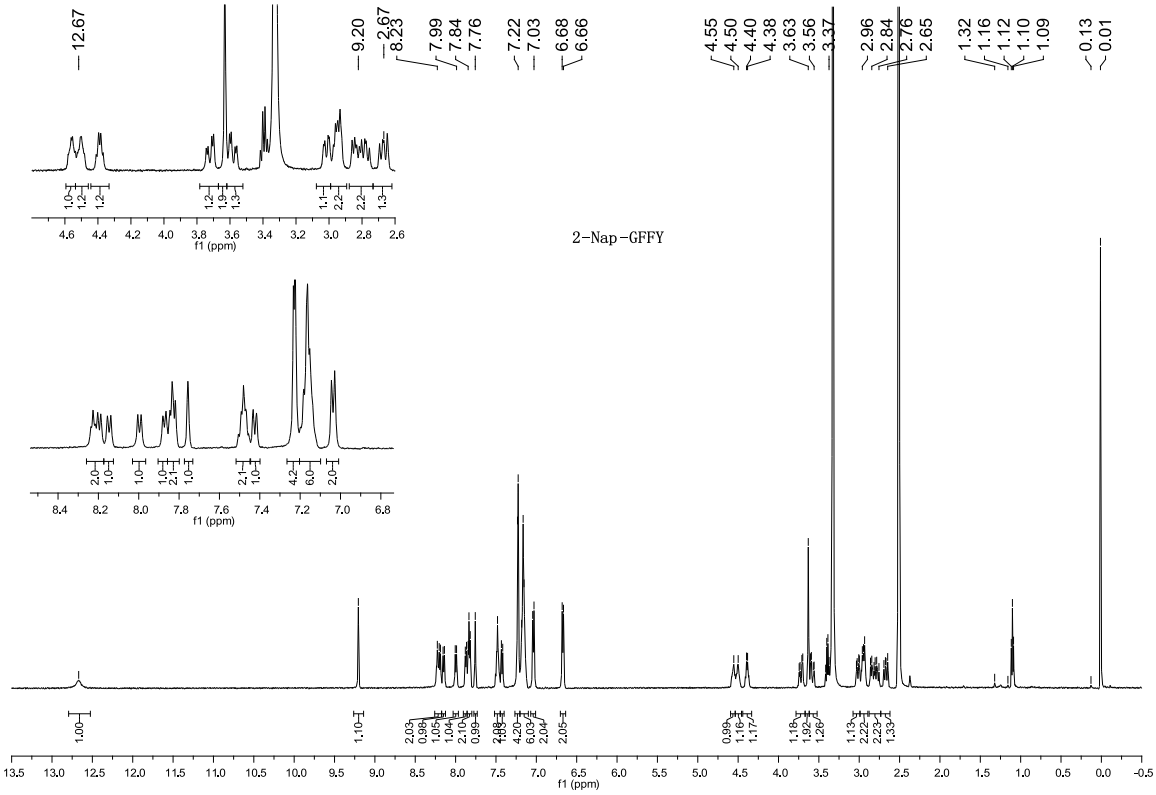
Synthesis of 2-NapGFFY (2-Nap-Gly-Phe-Phe-Tyr-OH)

The peptide was synthesized via Fmoc solid phase peptide synthesis. First, Fmoc-Tyr(^{t}Bu)-OH (1 eq.) and DIPEA (4 eq.) were dissolved in CH_2Cl_2 (20 mL g^{-1} resin). Then chlorotrityl chloride resin was added to the solution and the mixture was stirred at room temperature for 1 hr. The reaction was filtered and the unreacted resin was capped using DIPEA:MeOH:CH_2Cl_2 1:2:17 (v:v:v, 3 × 20 mL per gram resin). After the capping procedure, the resin was thoroughly washed with CH_2Cl_2, DMF, and CH_2Cl_2, and then dried over high vacuum. The bead loading was determined using 2% DBU/DMF method to be ~ 0.5 mmol g^{-1}. The beads were then swollen in DMF for 0.5 hr in a sealed spin column and DMF was then removed via filtration. 20% Piperidine/DMF (3 × 5 mL, 5 min each time) was added to remove the Fmoc protecting group. HBTU (4 eq.), Fmoc-Phe-OH or 2-Nap-Gly-OH (4 eq.), and DIPEA (8 eq.) were dissolved in DMF (~ 5 mL) and then transferred to the de-Fmoc resin. The mixture was shaken at room temperature for 2 hr for elongation of the sequence. Finally, 30% TFA in CH_2Cl_2 (20 mL g^{-1} resin) was incubated with the resin for ~ 3 hr at room temperature to cleave the peptide as well as to remove the protecting groups on the side chains of the amino acids. The volatile was removed and the residue was precipitated with diethylether. The product was collected by filtration and then dried over high vacuum. HRMS: (m/z)$[M+1]^+$ calcd. for $C_{41}H_{40}N_4O_7Na$: 723.2795, found: 723.2666. ^{1}H NMR (500 MHz, d_6-DMSO with TMS as the internal standard, rt): δ(ppm) 2.68 (1 H, dd, J_1= 12.5 Hz , J_2= 10 Hz), 2.81 (2 H, m), 2.95 (2 H, m), 3.02 (1 H, dd, J_1= 14 Hz , J_2= 5 Hz), 3.58 (1 H, dd, J_1= 15 Hz , J_2= 5 Hz), 3.63 (2 H, s), 3.72 (1 H, dd, J_1=15 Hz , J_2=5 Hz), 4.39 (1 H, m), 4.50 (1 H, m), 4.55 (1 H, m), 6.67 (2 H, d, J= 10 Hz), 7.04 (2 H, d, J= 10Hz), 7.16 (6 H, m), 7.23 (4 H, m), 7.42 (1 H, d, J= 5 Hz), 7.48 (2 H, m), 7.76 (1 H, s), 7.82 (2 H, m), 7.87 (1 H, d, J= 5Hz), 7.99 (1 H, J= 5 Hz), 8.15 (1 H, J= 5 Hz), 8.21 (2 H, m), 9.20 (1 H, s), 12.67 (1 H, s). The HPLC trace at 280 nm is shown below with t_R=17.2 min. HPLC conditions: GE SOURCE^{TM} 5RPC ST 4.6/150 column, solvent A: 0.1% TFA in CH_3CN, solvent B: 0.1% TFA in H_2O, gradient: 0 to 2.6 min, 1% A, 2.6 to 7.65 min, 1% to 5% A, 7.65 to 17.8 min, 5% to 99% A, 17.8 to 20.15 min, 99% to 100% A, 20.15 to 24 min, 100% to 99% A, 24 to 29 min, 99% A.



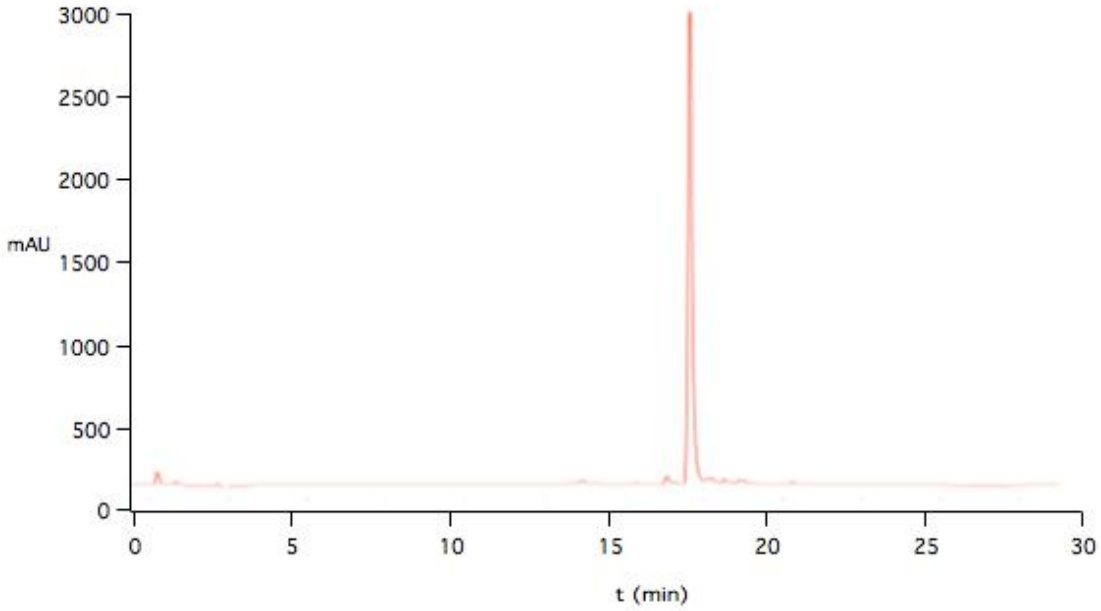


Figure S1 HPLC chromatogram of 2-NapGFFY.

Gel preparation

2-NapGFFY was dissolved H_2O in the presence of 1.04 eq. NaOH to make a final concentration of approximately 15 mg mL^{-1}. Then $K_2S_2O_8$ (1.4 eq.), H_2O, and SiO_2 nano-dispersed were added subsequently to the 2-NapGFFY solution to make 300 μL mixture containing 5 mg mL^{-1} (0.5 wt%) of peptide. The mixture was well mixed and then heated in a 95 ^oC water bath for ~ 5 min and then allowed to cool to room temperature for gelation.

After the gels were formed, they were subject to rheology measurement or the enzymatic reaction for further gelation. For the enzymatic gelation, 0.005 mg of mushroom tyrosinase (845 U mg^{-1}) was added to each microliter of gel and the gel was immediately shaked, spun down, and stored at room temperature without disturbance for gelation. We chose pH 6.6 for this study mainly because it is the optimum pH for mushroom tyrosinase. We have tested that mushroom tyrosinase also worked at pH 7.4 despite that the efficiency of the enzyme is a bit lower. Moreover, although the hydrogels prepared using this approach were at pH 6.6. We could adjusted the pH to 7.4 afterwards without jeopardize their mechanical properties.

DOPA is a bioactive molecule with very important therapeutic applications (e.g. clinical treatment of Parkinson's disease). The pros and cons of the hydrogel containing DOPA should be carefully evaluated before its applications as biomedical materials.

After 10 hr, the gel was transferred to the Haake RheoStrss 6000 rheometer plate for measurement.

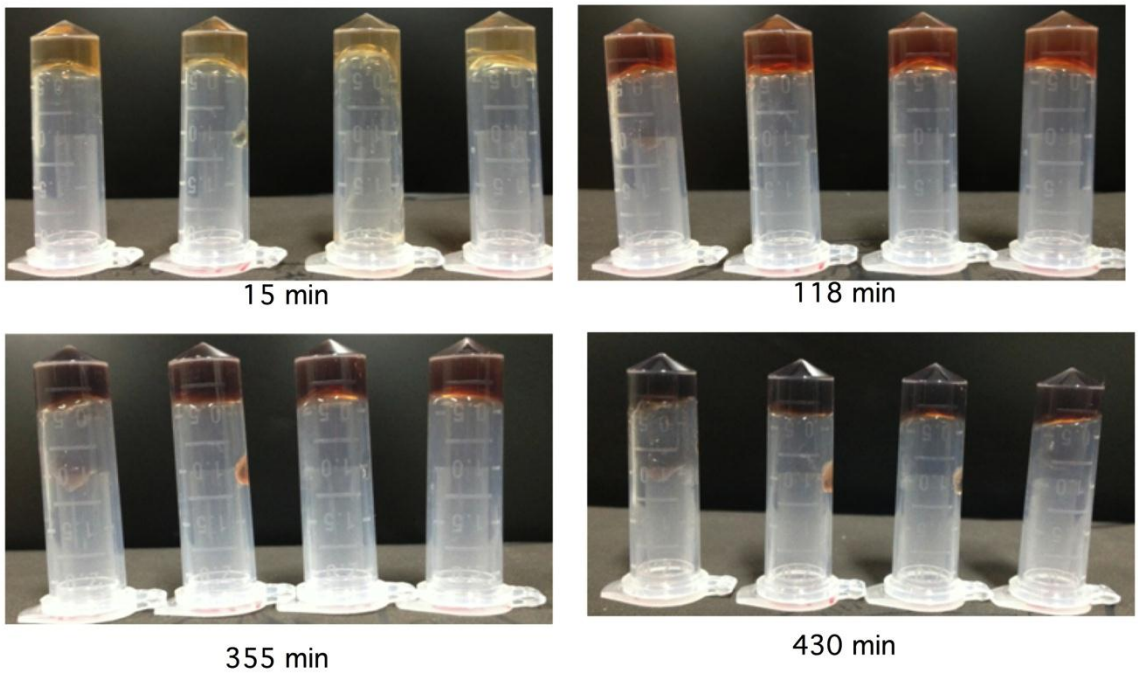


Figure S2 The gel images for the enzymatic gelation at different time. For each image, the reaction time was indicated and from the left to the right, the gels contained 0%, 20%, 50%, and 100% of SiO_2 nanoparticles (weight ratio relatively to that of the peptide).

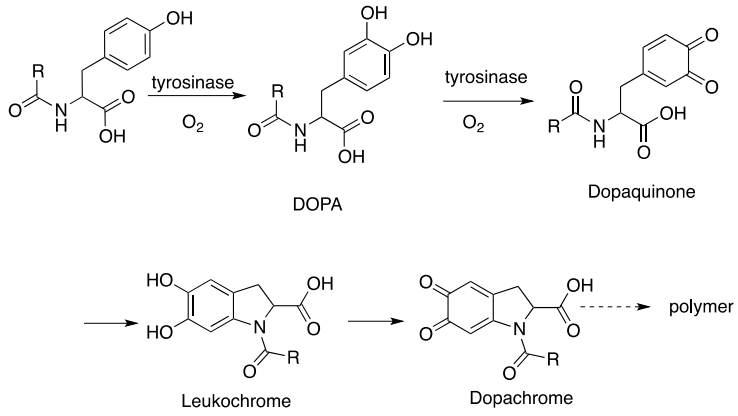


Figure S3 Tyrosinase catalyzed reaction scheme.

Rheology experiment

The hydrogel was carefully loaded to the rheometer plate using a spatula. The rheology experiments were then carried out with an oscillation time sweep mode at 1 Hz and/or a frequency-sweep mode at 0.1% strain on a Haake RheoStrss 6000 rheometer (geometry: 1^{o}/20 mm of cone and plate).

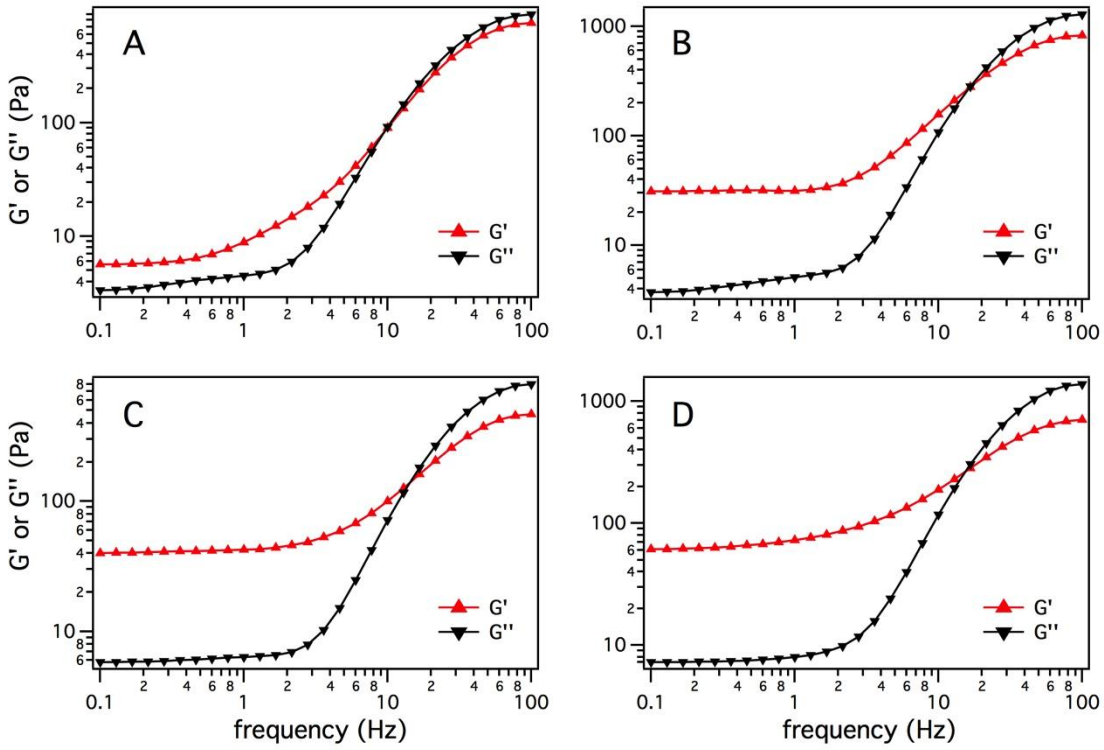


Figure S4 Rheological measurements in the dynamic frequency sweep mode for gels of 0.5 wt% without enzyme treatment. A contains no SiO_2 nanoparticles; B contains 0.1 wt% of SiO_2 nanoparticles (20%); C contains 0.25 wt% of SiO_2 nanoparticles (50%); and D contains 0.5 wt% of SiO_2 nanoparticles (100%).

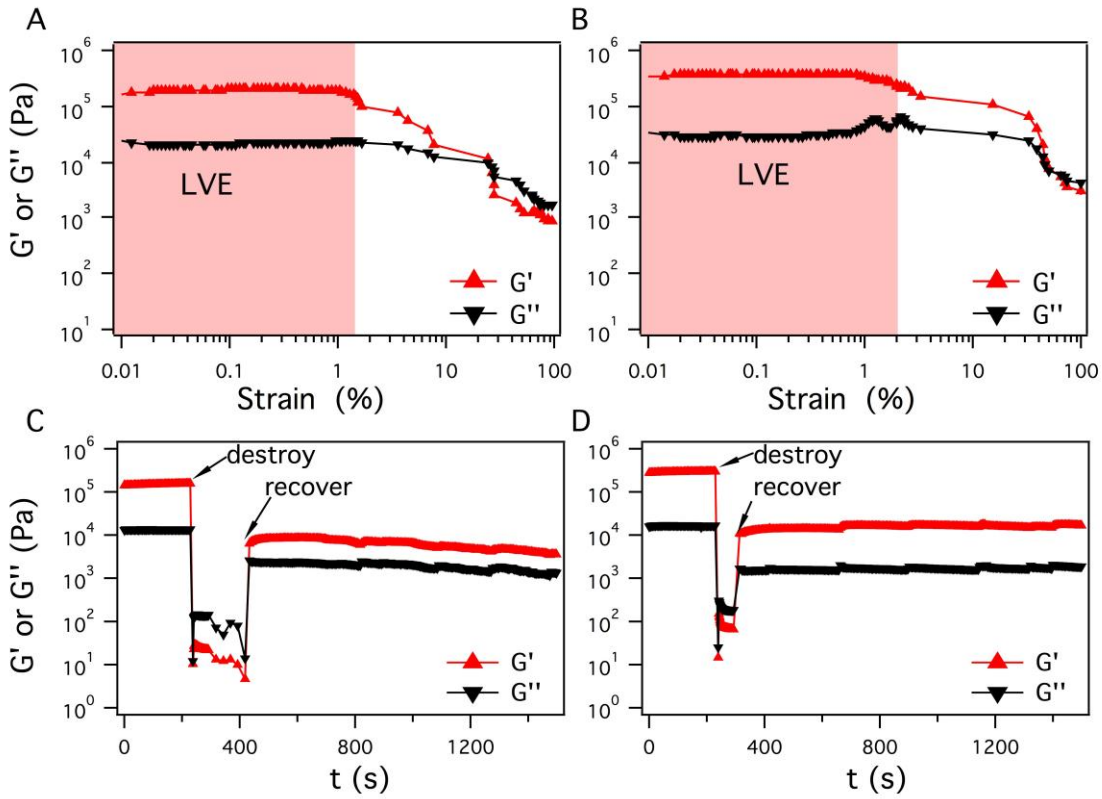


Figure S5 The strain-sweep (A and B) and recovery (C and D) experiments on the EANC gels (5 mg mL^{-1}). A and C contain no SiO_2 nanoparticles; B and D contain 0.5 wt% of SiO_2 nanoparticles (100%). The strain-sweep experiments were carried out at 1 Hz from strain amplitude range of 0.01% to 100%. The linear viscoelastic (LVE) regions are shaded in light pink. The recovery experiments were carried out with an initial oscillation time sweep at 1 Hz and 0.1% strain for 240 s, the gel was then destroy by 100% strain amplitude at 100 Hz for 60 s, and the recovery status was measure immediately by the oscillation time sweep at 1 Hz and 0.1% strain for 1200 s. The points, at which the gel started to break and at which the gel started to recover, are noted by arrows respectively in the figures.

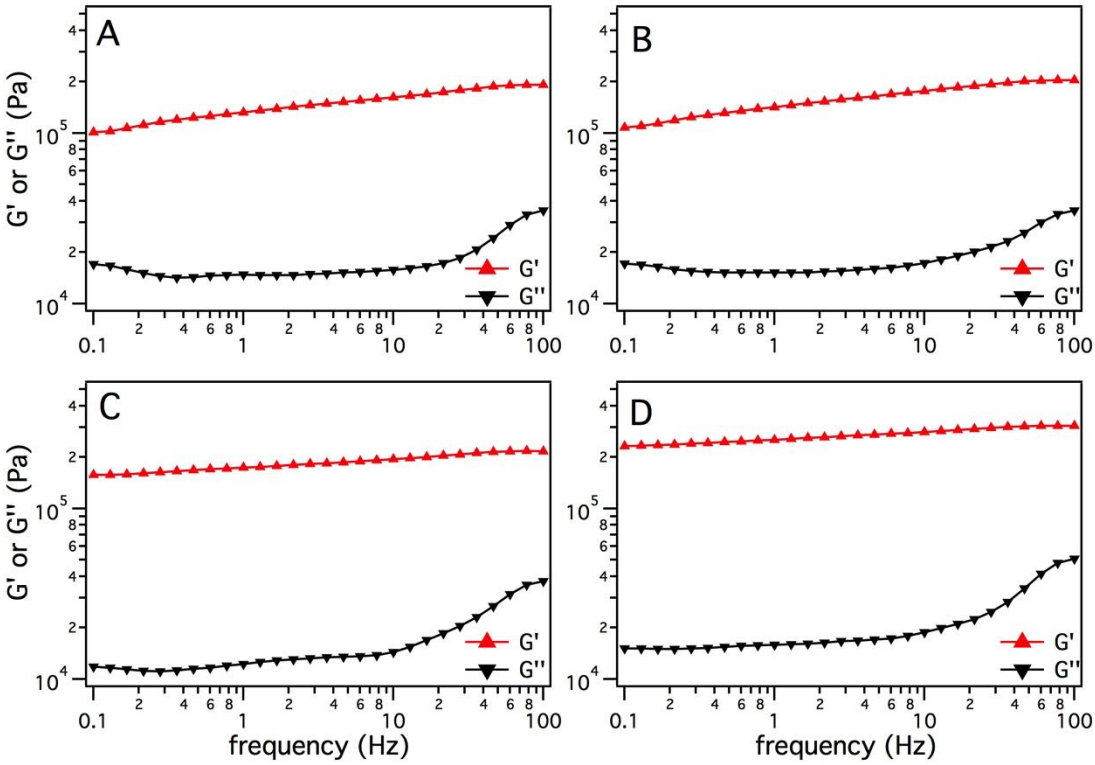


Figure S6 Rheological measurements in the dynamic frequency sweep mode for gels of 0.5 wt% treated with mushroom tyrosinase. A contains no SiO_2 nanoparticles; B contains 0.1 wt% of SiO_2 nanoparticles (20%); C contains 0.25 wt% of SiO_2 nanoparticles (50%); and D contains 0.5 wt% of SiO_2 nanoparticles (100%).

Enzymatic reaction monitored by UV-Vis spectroscopy

The enzymatic reaction of 2-NapGFFY was monitored by UV-Vis spectroscopy with JASCO V500 UV-Vis spectrophotometer, Japan to monitor the reaction process. Briefly, a stock solution of mushroom tyrosinase (10 mg mL^{-1}, 8.4 U μL^{-1}) and that of 2-NapGFFY (3.8 mg mL^{-1}) in 50 mM phosphate buffer (pH 6.6) were prepared. Then 5 μL of each were mixed to initiate the reaction at different time points. At the end of this experiment, all reactions were diluted with 800 μL of H_2O to quench the reaction and their UV-Vis spectra were recorded immediately on a JASCO V500 UV-Vis spectrophotometer. Those for the same concentration of 2-NapGFFY and mushroom tyrosinase were also recorded for analysis. The result was shown in Figure 4b and Figure S7 respectively.

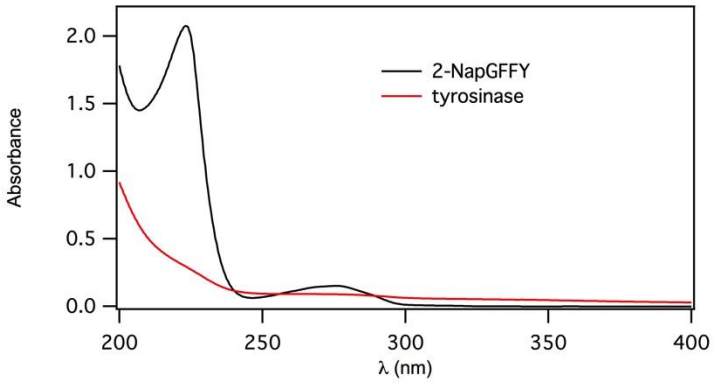


Figure S7 The UV-Vis spectra of 2-NapGFFY and tyrosinase in the range of 400 nm -200 nm.

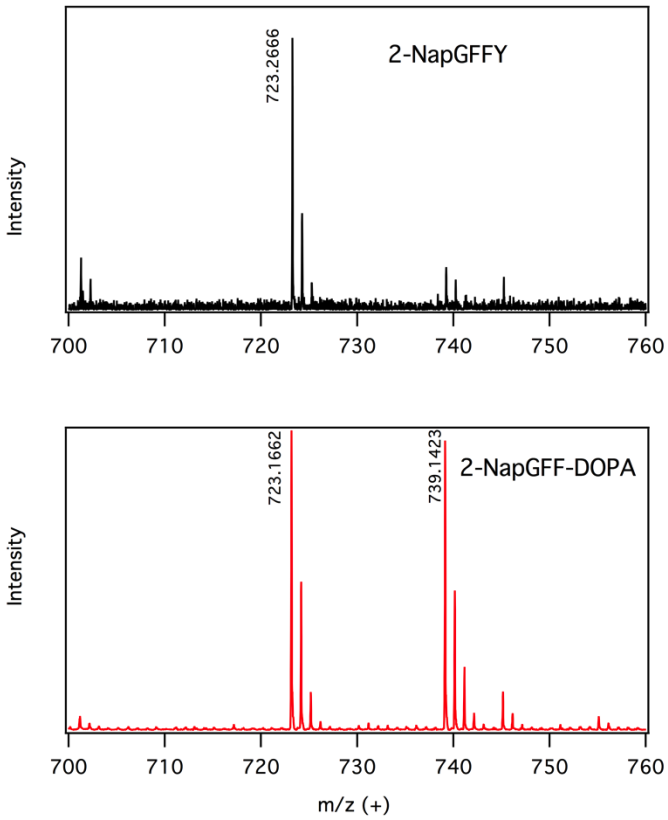


Figure S8 Mass spectra of 2-NapGFFY and the crude enzymatic reaction.

Atomic force microscopy (AFM) experiment

The microscopic morphologies of the hydrogels were imaged at room temperature by a NanoWizard II AFM (JPK, Germany) operating in intermittent contact (Air) mode (conditions: scan rate, 1 Hz; pixel number, 512 × 512). Silica cantilevers (AC160, Olympus, Japan) with typical tip radii of ~10 nm and resonance frequencies of ~200 to 500 kHz were used for imaging. The samples were prepared as describe: 20 μL the hydrogel was resuspended in 480 μL H_2O and mixed well. Then 50 μL the newly prepared solution was loaded to the surface of a freshly peeled mica surface and allowed

to adsorb for 5 min. The abundant solution was carefully removed and the mica surface was allowed to dry at room temperature and the plate was further blew with air to remove any loosely attached materials prior to the AFM measurement.

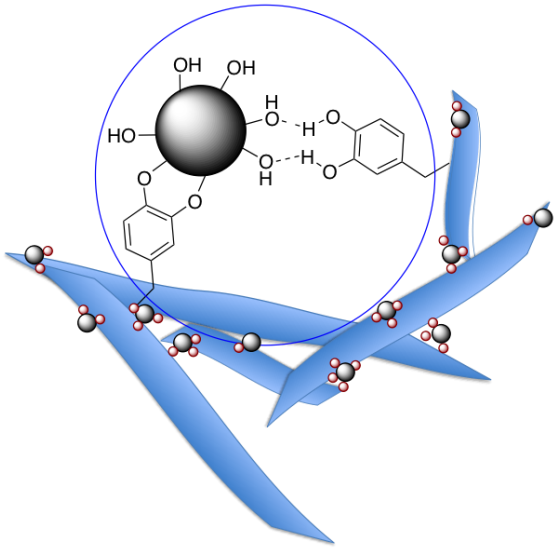


Figure S9 Proposed mechanism for the NP enhanced hydrogel properties by hydrogen bonding and weakly covalent bonds.