Supporting Information

Peroxidase-Mediated *in situ* Fabrication of Multi-Stimuli- Responsive and Dynamic Protein Nanogels from Tyrosine-Conjugated Biodynamer and Ovablumin

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Experimental section

Materials. Di(ethylene glycol)ethyl ether acrylate (DEGA, Sigma, 98%) and poly(ethylene glycol) methyl ether acrylate (PEGA, M_n 480 g·mol^{-1}, Sigma) were purified by passing through a basic alumina column. L-Tyrosine hydrazide (98%), reduced L-glutathione (GSH, 98%), tris(2-carboxyethyl) phosphine hydrochloride (TCEP, 98%) and dansyl hydrazine (98%) were purchased from Sigma-Aldrich. Ovalbumin (OVA, 90%) and curcumin (≥95%) were purchased from Dingguo Biotechnology Co. Ltd. Horseradish peroxidase (HRP) were purchased from Sangon Biotech (Shanghai) Co. Ltd. All the above chemicals were used as received. Azobis(isobutyronitrile) (AIBN) was recrystallized twice from methanol. The bisaldehyde-functionalized RAFT agent (CTA-bisCHO) was prepared according to the method reported in our previous paper.[1] All other reagents were commercial chemicals and used directly, except those specially claimed. All solvents were redistilled before use.

Characterization. ^{1}H NMR measurements were recorded on a Varian UNITY- plus 400 M nuclear magnetic resonance spectrometer using $CDCl_3$ or DMSO as the solvent. The number-average molecular weight (M_n), weight-average molecular weight (M_w), and polydispersity ($Đ$) of the synthesized polymers were determined by GPC at 35°C with a Waters 1525 chromatograph equipped with a Waters 2414 refractive index detector, where tetrahydrofuran (THF) was used as eluent at a flow rate of 1 mL/min and polystyrene standards were used for calibration. UV-vis spectroscopy was performed on a Shimadzu UV-2450 UV-vis spectrophotometer. Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorophotometer. Dynamic light scattering (DLS) analyses were conducted on a Zetasizer Nano ZS from Malvern Instruments equipped with a 10 mW HeNe laser at a wavelength of 633 nm at a 90° angle. Transmission electron microscopy (TEM) observations were carried out on a Tecnai G2 20 S-TWIN electron microscope equipped with a Model 794 CCD camera. The samples were deposited on a carbon-coated copper grid and water was evaporated in air. To increase the contrast, the samples were stained with hydrazine vapor and OsO_4 vapor.

Preparation of bisaldehyde-functionalized copolymer P(DEGA-co-PEGA)-bisCHO (CP-bisCHO). The copolymer P(DEGA-*co*-PEGA) was synthesized by reversible addition-fragmentation chain transfer (RAFT) radical polymerization using a bisaldehyde-functionalized RAFT agent.[2] Briefly, DEGA (0.50 g, 2.7 mmol), PEGA (0.32 g, 0.67 mmol), CTA-bisCHO (85.9 mg, 0.133 mmol) and AIBN (2.2 mg, 0.013 mmol) were dissolved in 2 mL of DMF in a flask. The solution was degassed by three freeze-pump-thaw cycles. The polymerization was carried out at 60°C for 24 h, and then stopped by cooling the solution in ice water and exposing the solution to air. The P(DEGA-co-PEGA) copolymer was precipitated into *n*-hexane, centrifuged, and dried overnight under vacuum at room temperature. To eliminate the influence of thiocarbonylthio group at ω-terminus on the following reactions, the copolymer reacted with excess AIBN at 100°C to replace the thiocarbonylthio group with 2-cyanopropyl according to the method reported by Tang et al.[3] P(DEGA-*co*-PEGA) (0.2 g, 33 μmol), AIBN (0.54 g, 3.3 mmol) and DMF (20 mL) were added into a flask. The flask was degassed by three freeze-pump-thaw cycles. Then the reaction solution was stirred at 100 °C for 12 h. The modified copolymer P(DEGA-*co*-PEGA)-bisCHO (CP-bisCHO) was isolated by precipitating into *n*-hexane and dried overnight under vacuum at room temperature.

Preparation of tyrosine-conjugated biodynamer CP-*dyn*-Tyr via acylhydrazone linkages. CP-bisCHO (100 mg, 15 mmol) and L-tyrosine hydrazide (58.5 mg, 0.30 mmol) were dissolved in 5 mL of DMF. Using trifluoroacetic acid (TFA) as the catalyst, the reaction was carried out at 60 °C for 24 h. The excess tyrosine hydrazide was removed by dialysis against $NaHCO_3$ solution (0.01 M) for 1 day and distilled water for 2 days (MWCO 1000). The purified tyrosine-bioconjugated dynamic covalent copolymer CP-*dyn*-Tyr was recovered by lyophilization.

Preparation of biodynamer nanoparticles via HRP-catalyzed tyrosine coupling reaction. A typical procedure to prepare the cross-linked CP-*dyn*-Tyr nanoparticles was described as follows. The biodynamer CP-*dyn*-Tyr and HRP were dissolved in 1 M Tris-HCl (pH 8.0) at final concentrations of 5.0 mg/mL and 0.05 mg/mL, respectively. The coupling reaction of CP-*dyn*-Tyr was initiated by adding H_2O_2 (30

wt%) solution to the mixture 5 times every 10 min to a final concentration of 50 μM. After 24 h reaction at 37 °C, the nanogels were purified by dialysis against deionized water for 2 days (MWCO 1000). An aliquot of the solution was lyophilized to obtain dried nanoparticles for characterization.

Preparation of biodynamer-protein nanogels by HRP-mediated coupling reaction. HRP-catalyzed cross-linking reactions of the biodynamer and OVA were carried out at various concentrations of OVA and a fixed concentration of CP-*dyn*-Tyr (5 mg/mL)in 1 M Tris buffer (pH 8.0) at 37 °C. Typically, CP-*dyn*-Tyr (5 mg/mL), OVA (1, 2, 5 or 7 mg/mL) and HRP (0.05 mg/mL) were dissolved in 1 M Tris-HCl (pH 8.0). The reaction of CP-*dyn*-Tyr and OVA was initiated by adding H_2O_2 (30 wt%) solution to the mixture 5 times every 10 min to a final concentration of 50 μM. The reaction mixture was incubated at 37 °C during the addition of H_2O_2 and for a further 24 h following the final addition of H_2O_2. The protein nanogels were purified by dialysis against deionized water for 1 day and ultrafiltration (MWCO 100 kDa). The yield of OVA nanogels were are 29 (1 mg/mL OVA), 55 (2 mg/mL OVA), 70 (5 mg/mL OVA) and 85 wt% (7 mg/mL OVA).

pH-Triggered cleavage of acylhydrazone bonds in protein nanogels. The pH-triggered cleavage reaction was conducted at two pH values: pH 5.0 and pH 7.4. Typically, the lyophilized OVA-NG5 nanogels (5 mg) were dissolved in the aqueous solution (1 mL, pH 5.0 or 7.4). The solution was stirred at room temperature, the samples were taken at specified intervals and analyzed by SDS-PAGE.

Chain exchange reaction of protein nanogels with dansyl hydrazine. The chain exchange reactions of protein nanogels with dansyl hydrazine were carried out at 25 °C and 37 °C. Typically, the lyophilized OVA-NG5 nanogels (5 mg, 0.83 μmol) was dissolved in deionized water (pH 5.0, 1 mL), the solution of dansyl hydrazine (2.21 mg, 2 μmol) in DMF (100 μL) was added dropwise. After 24 h stirring, the solution was dialyzed against distilled water for 2 days (MWCO 1000), and the chain exchange product was recovered by lyophilization. The amount of dansyl hydrazine participated in chain exchange reaction was measured by fluorescence spectroscopy.

Preparation of curcumin(Cur)-loaded protein nanogels. CP-*dyn*-Tyr, OVA (rOVA) and HRP were dissolved in 1 M Tris-HCl (pH 8.0) at final concentrations of 5 mg/mL, 5 mg/mL and 0.05 mg/mL, respectively. The solution of curcumin in acetone (10 mg/mL) was added dropwise with a final concentration of 0.5 mg/mL. The cross-linking reaction was initiated by adding H_2O_2 (30 wt%) solution to the mixture 5 times every 10 min to a final concentration of 50 μM. The reaction mixture was incubated at 37 °C during the addition of H_2O_2 and for a further 24 h following the final addition of H_2O_2. The reaction mixture was purified by dialysis against distilled water for 1 day (MWCO 12 kDa), and was centrifuged to remove insoluble curcumin. The entrapped efficiency (EE) and the loading content (LC) of curcumin in the protein nanogels were determined by a UV-Vis spectrophotometer at 428 nm. The EE and LC were calculated according to the following equations:

EE (%) = (amount of loaded Cur)/(total amount of feeding Cur) ×100%

LC (%) = (amount of loaded Cur)/(amount of Cur-loaded nanogels) × 100%

Release of curcumin from protein nanogels. The in vitro release of curcumin from protein nanogels was performed in phosphate buffer saline (0.1 M, pH 7.4) and acetate buffer saline (0.1 M, pH 5.0) containing 0.1% w/v Tween-80 with/without GSH at 37 °C. By addition of Tween-80, the buffer solutions not only maintain a sink condition, but also provide solubility for curcumin in aqueous phase. In a typical release experiment, the aqueous solution of curcumin-loaded protein nanogels (1 ml) was mixed with four equal volume of buffer solution (pH 7.4 or 5.0) and then the solution was transferred to a dialysis tube with a MWCO 3500. The dialysis tube was immersed into 20 mL of corresponding buffer solution and gently shaken at 37 °C. At specified intervals, 5 mL of the release medium was taken out and replaced with an equal volume of fresh medium. The release amount of curcumin with time was determined by a UV-vis spectrophotometer at 428 nm. The release experiments were conducted in triplicate.

LCST measurement. The turbidity of the polymer solutions was determined at $\lambda = 600$ nm using a Shimadzu UV-2450 UV-visible spectrophotometer equipped with a temperature control unit. The concentration was kept at 0.2 wt%. The LCST was

defined as the temperature inducing a 10% decrease in the original optical transmittance.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed with 10% polyacrylamide gels. Electrophoresis was carried out at 80 V voltage, 16 mA current for 4 h. Staining was accomplished using Coomassie Brilliant Blue R-250 solution.

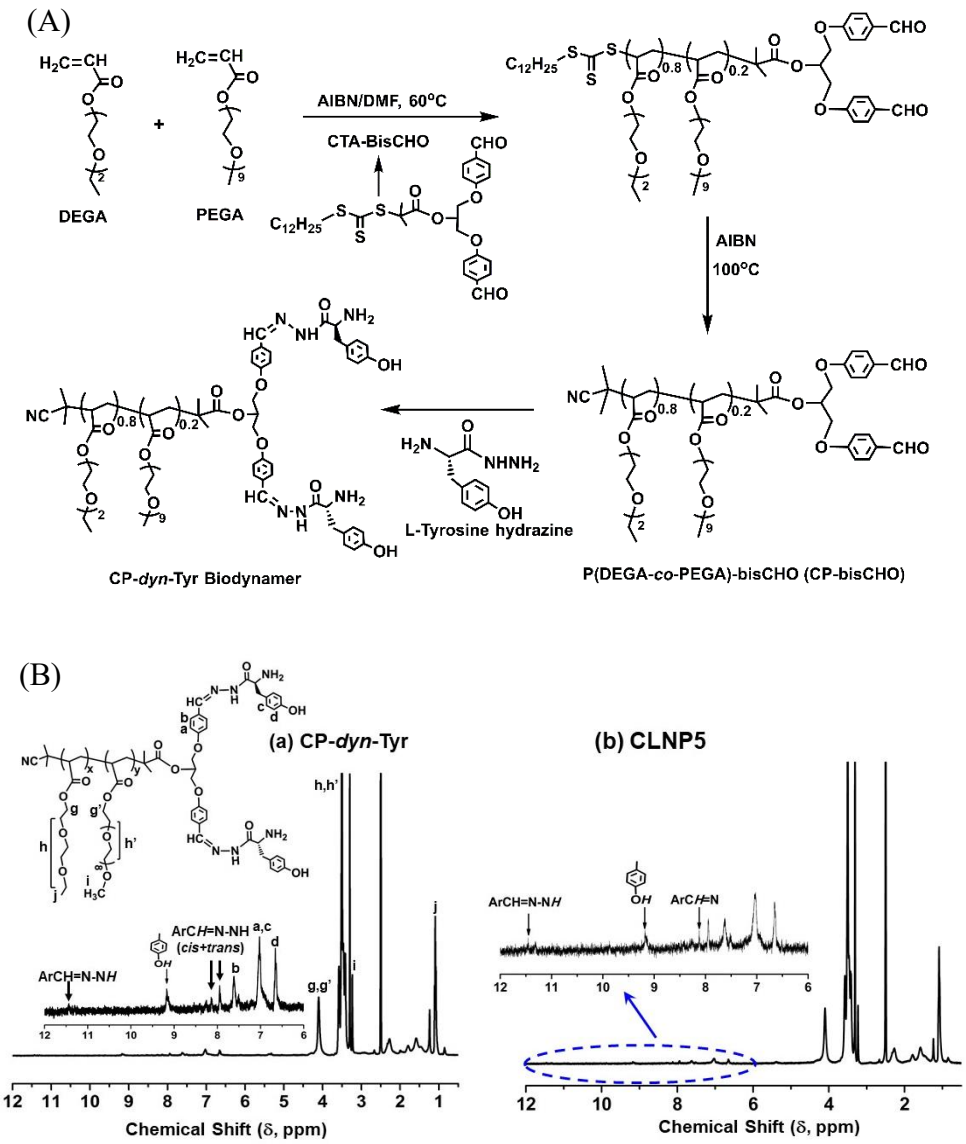


Figure S1. (A) Illustration of synthesis of tyrosine-conjugated biodynamer CP-*dyn*-Tyr. (B) ^{1}H NMR spectra of CP-*dyn*-Tyr and the cross-linked nanoparticles.

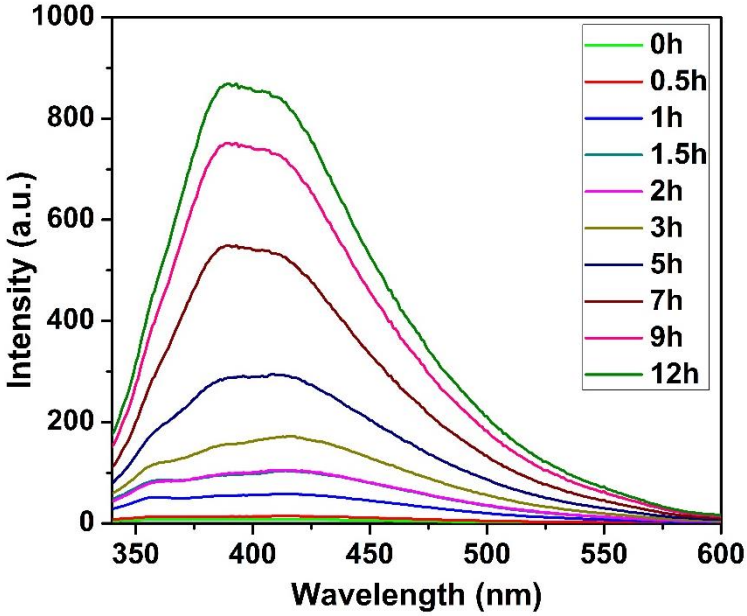


Figure S2. Time-dependence of fluorescence emission spectra of CP-*dyn*-Tyr in the presence of HRP (0.05 mg/mL) and H_2O_2 (50 μM) in 1 M Tris buffer (pH 8.0) at 37 °C.

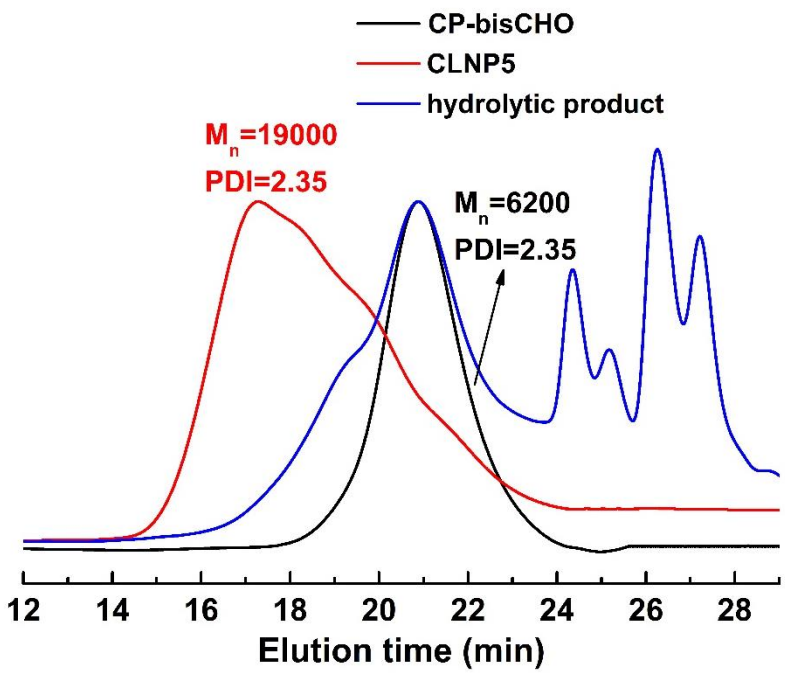
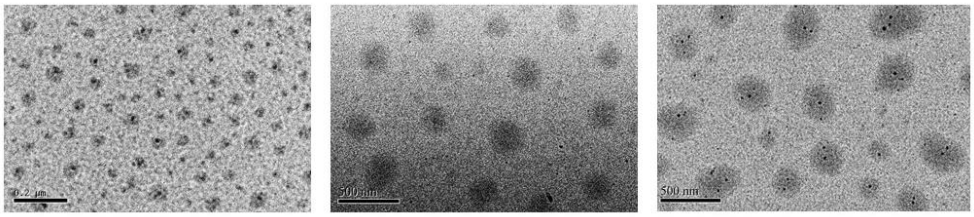


Figure S3. GPC curves of CP-*dyn*-Tyr biodynamer, the cross-linking product and the hydrolytic specimen at pH 3.0 and 37°C.



(a) 2 mg/mL (b) 7 mg/mL (c) 10 mg/mL

Figure S4. TEM images of CLNP nanoparticles prepared at various concentration of CP-*dyn*-Tyr: (a) 2 mg/mL, (b) 7 mg/mL and (c) 10 mg/mL.

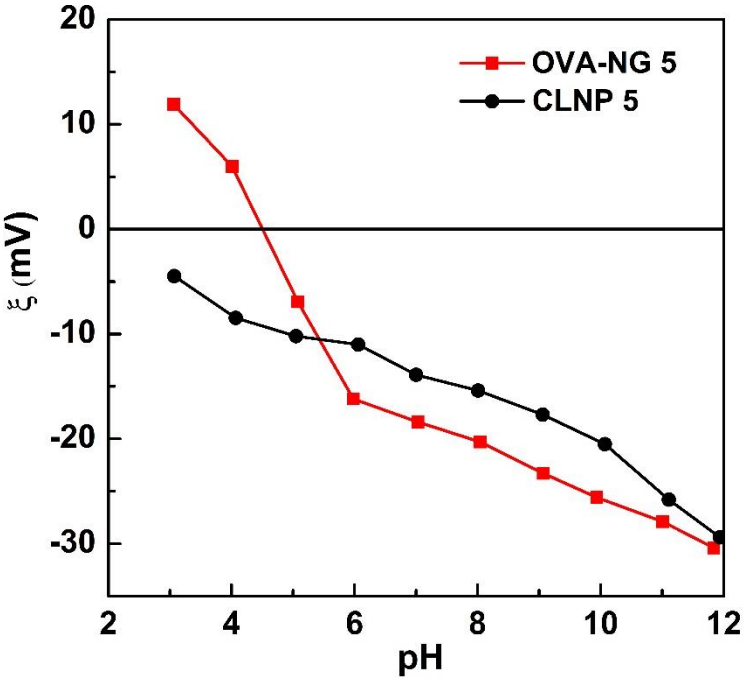


Figure S5. Dependence of Zeta potential of OVA-NG5 and CLNP5 on pH.

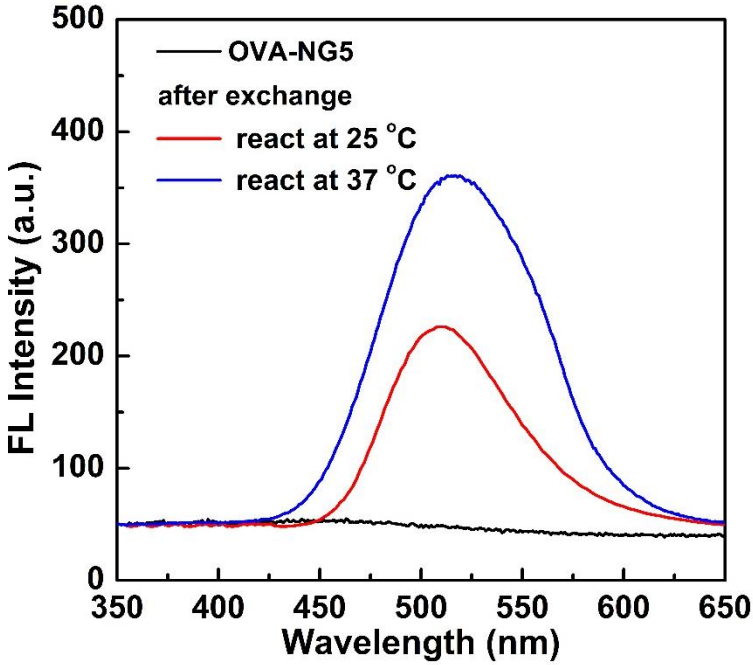


Figure S6. Fluorescence spectra of OVA-NG5 nanogels before and after chain exchange reaction with dansyl hydrazine at various temperatures under pH 5.0.

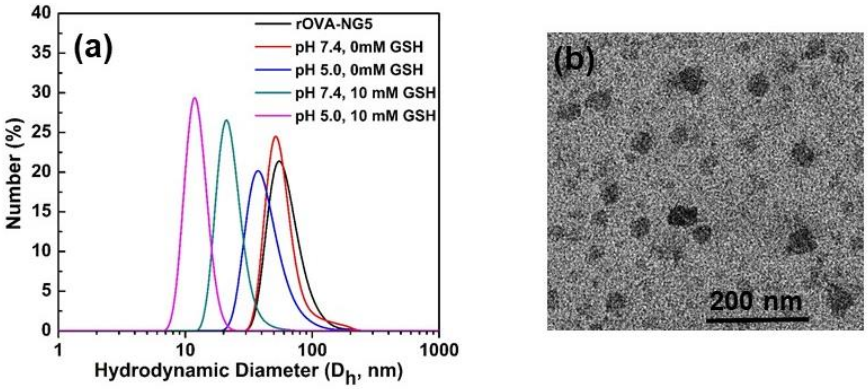


Figure S7. (a) DLS curves of rOVA-NG5 nanogels before and after 24 h incubation at 37 °C under various conditions. (b) TEM image of rOVA-NG5 nanogels.

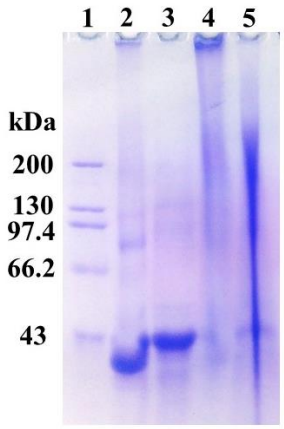


Figure S8. SDS-PAGE analysis results: line 1: protein maker; line 2&3: OVA; line 4&5: rOVA-NG5 nanogels. (line 2, 4: without DTT; line 3, 5: the specimens were treated with TCEP).

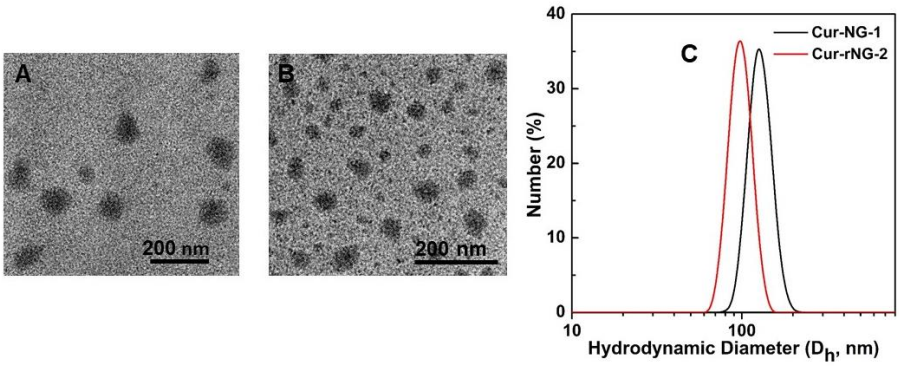


Figure S9. (A&B) TEM images of Cur-loaded protein nanogels Cur-NG-1 (A) and Cur-rNG-2 (B). (C) DLS curves of Cur-loaded protein nanogels Cur-NG-1 and Cur-rNG-2.

References:

(1) Li, J. Y.; Yang, S. X.; Wang, L.; Wang, X. B.; Liu, L. Thermoresponsive Dynamic Covalent Polymers with Tunable Properties. *Macromolecules* **2013,** *46,* 6832-6842.

(2) Wang, L.; Liu, L.; Wu, L. B.; Liu, L. Z.; Wang, X. B.; Yang, S. X.; Zhao, H. Y. Environmentally Responsive Amino Acid-Bioconjugate Dynamic Covalent

Copolymer As a Versatile Scaffold for Conjugation. *RSC Adv.* **2015,** *5*, 30456-30463.

(3) Chong, Y. K.; Moad, G.; Rizzardo, E.; Thang, S. H. Thiocarbonylthio End Group Removal from RAFT-Synthesized Polymers by Radical-Induced Reduction. *Macromolecules* **2007,** *40*, 4446-4455.