Supporting Information for

Efficient Way to Generate Protein-Based Nanoparticles by in-Situ Photoinitiated Polymerization-Induced Self-Assembly

Chao Ma, Xiaoman Liu, Guangyu Wu, Pei Zhou, Yuting Zhou, Lei Wang and Xin Huang**

MIIT Key Laboratory of Critical Materials Technology for New Energy Conversion and Storage, State Key Laboratory of Urban Water Resource and Environment, School of Chemistry and Chemical Engineering, Harbin Institute of Technology, Harbin, 150001, China

E-mail: *liuxiaoman@hit.edu.cn; xinhuang@hit.edu.cn*

Experimental Section

Materials

4,4’-azobis(4-cyanovaleric acid) (ACVA, ≥ 98%, Sigma Aldrich), 2-mercaptothiazoline (≥ 98%, Sigma Aldrich), N’-dicyclohexylcarbodiimide (DCC, ≥ 99%, Fluka), 4-(dimethylamino)pyridine (DMAP, ≥ 99%, Sigma Aldrich), carbon disulfide (≥ 99%, Sigma Aldrich), bovine serum albumin (BSA, ≥ 98%, Sigma Aldrich) were used without further purification. 2-Hydroxypropyl methacrylate (HPMA, ≥97%, Energy Chemical) was purified by passing through an alumina column to remove inhibitors. Tris(2,2′-bipyridyl) dichlororuthenium (II) hexahydrate ($[Ru(bpy)_3Cl_2 \cdot 6H_2O]$, ≥ 99.5%，Sigma Aldrich), pyrene (≥ 97 %，Aladdin)，4-nitrophenyl acetate (≥ 98%，Aladdin), Nile Red (≥ 95%, Energy Chemical), doxorubicin hydrochloride (DOX, ≥ 98 %，Aladdin), deoxyribonucleic acid (DNA, ≥98%，Sigma Aldrich), SYBR Green I (≥ 98%，Sigma Aldrich)，protease (Sigma Aldrich) were used as received.

1. Synthesis of mercaptothiazoline activated trithiol RAFT agent[1]

1.1. Synthesis of mercaptothiazoline activated 4,4’-azobis(4-cyanovaleric acid)(ACVA-ACPM).

The general procedure was shown in supplementary Figure S1. A solution of 4,4’-azobis(4-cyanovaleric acid) (ACVA, 6.01 g, 21.5 mmol) and 2-mercaptothiazoline (6.75 g, 56.7 mmol) in 1,4-dioxane (200 mL) was degassed by nitrogen for 30 min. N,N’-dicyclohexylcarbodiimide (DCC, 10.23 g, 49.6 mmol) and 4-(dimethylamino)pyridine (DMAP, 0.15 g, 1.23 mmol) dissolved in 1,4-dioxane (100 mL) were added slowly at room temperature under rigorous stirring. After 20 h, the reaction mixture was filtered and concentrated. The product was precipitated in cold diethyl ether. After drying under vacuum, ACVA-ACPM was obtained as a yellow powder (7.20 g, 69% yield).

1.2 Synthesis of bis(propylsulfanylthiocarbonyl) disulfide.

Propanethiol (8.69 g, 114 mmol) was added over 10 min to a stirred suspension of sodium hydride (60% in oil) (4.73 g, 118.5 mmol) in diethyl ether (150 mL) at 0 °C. The solution was then allowed to stir for 10 min prior to the addition of carbon disulfide (9.0 g, 118.5 mmol). Crude sodium S-propyl trithiocarbonate (11.78 g, 73.5 mmol) was collected by filtration, suspended in diethyl ether (100 mL), and reacted

with iodine (9.45 g, 0.038 mol). After 1 h the solution was filtered, washed with aqueous sodium thiosulfate, and dried over sodium sulfate. The crude bis(propylsulfanylthiocarbonyl) disulfide was then isolated by rotary evaporation.

1.3 Synthesis of mercaptothiazoline activated trithiol-RAFT agent 4-cyano-4-(propylsulfanylthiocarbonyl) sulfanylpentanoic acid mercaptothiazoline amide.

A solution of bis(propylsulfanylthiocarbonyl) disulfide (1.37 g, 5.0 mmol) and ACVA-ACPM(4.5 g, 11.3 mmol) in ethyl acetate (50 mL) was heated at reflux for 18 h. Following rotary evaporation of the solvent, the crude product was isolated by column chromatography using silica gel as the stationary phase and 50:50 ethyl acetate/hexane as the eluent (2.56 g, 41% yield). ^{1}H NMR was shown in Figure S2.

2. Synthesis of 4-cyano-4-(propylsulfanylthiocarbonyl) sulfanylpentanoic acid mercaptothiazoline amide anchored BSA (BSA-CTA).

The synthesis of BSA-CTA was conducted in solvent of water/DMSO mixture (9/1, v/v) at room temperature. 2.9 mg (7.6×10^{-3} mol) of mercaptothiazoline-activated trithiol-RAFT agent was dissolved in 3 mL of DMSO, and the solution was added dropwisely to a stirred solution of BSA (50 mg, 7.6×10^{-4} mol, in 27 mL of water) to give a trithiol-RAFT agent: protein molar ratio of 10:1. The mixed solution was stirred for 20 h, and then purified by dialyzing (dialysis tubing with MWCO of 8–14 kDa) extensively against deionized water. Finally, the solution was freeze-dried to yield a white powder. And the obtained BSA-CTA was characterized by SDS–PAGE, MALDI-MS, DLS and UV–vis spectroscopy.

3. Determination of the BSA-CTA by UV–vis spectroscopy

To determine the composition of BSA-CTAs, a standard trithiocarbonate absorbance curve was performed based on the trithiol-RAFT agent by monitoring the chromophore corresponding to the RAFT at 320 nm (Figure S4). According to the Lambert-Beer Law： $A = \varepsilon bc$. We got the value of ε (8985.6 L/mol•cm). Then, c_{BSA}/ c_{RAFT} could be calculated to clarify the composition of BSA-CTA with the formula: $c_{BSA}\cdot V\cdot M_{BSA} + c_{RAFT}\cdot V\cdot M_{RAFT} = m_{BSA\text{-}CTA}$ (Table S1).

4. Synthesis of BSA-PHPMA nanoparticles based on photo-initiated RAFT polymerization-induced self-assembly.

The reaction was conducted at 5 % (w/w) solids content. 10 mg of BSA-CTA

(1.5×10^{-4} mmol) was dissolved in 1 mL of water. Then HPMA monomer (50 mg, 0.35 mmol) and $[Ru(bpy)_3Cl_2\cdot6H_2O]$ aqueous solution was added slowly to the BSA-CTA solution. The final concentration ratios were as follows: $[HPMA]:[BSA\text{-}CTA_8]:[[Ru(bpy)_3Cl_2\cdot6H_2O]]=2350:8:0.27$. The solutions were purged with argon gas for 10 min. The reaction vessels were irradiated under blue LED light (460 nm) at room temperature. Aliquots were taken at predetermined time intervals. These samples were directly analyzed by TEM, SEM, GPC, DLS and UV–vis spectroscopy.

In addition, the synthesis of BSA- PHPMA nanoparticles at 15 % solids content followed the same procedure as above, except changing the amount of monomer employed, and using another RAFT agent $BSA\text{-}CTA_5$ as an initiator.

5. Measurement of the catalytic activity of BSA after polymerization.

The activity of BSA was conducted according to published procedure.[2-5] 10 μL of 4-nitrophenyl acetate acetonitrile solution (10 mM) was add to 100 μL of BSA or BSA-PHPMA solution ([BSA] = 0.3 mM) in phosphate buffer (pH 8.0), and then 900 μL of phosphate buffer (pH 8.0) was added to the mixture and the temperature of the reactor is kept at 25 °C for 20 min. The absorbance of the samples was determined at 405 nm.

6. MTT Assay.

The cell viability of NIH 3T3 cells in presence of BSA-PHPMA nanoparticles was determined using MTT method.[6] Cells were seeded in a 96-well plate with a density of 1.0×10^4 cells per well in 150 μL of culture medium. After 12 h incubation, the medium was replaced with 150 μL of medium containing serial dilutions (0.5, 2.5, 5, 7.5, 10, 15 mg/mL) of BSA-PHPMA nanoparticles. The cells were incubated (37 °C, 5 % CO_2) for another 24 h and 36 h, respectively, then the medium was replaced with a solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphen-yl-2H-tetrazolium bromide (MTT) in medium (100 μL, 1.0 mg/mL),[7] followed by 4 h incubation. The medium was then replaced by DMSO (100 μL/well). The absorbance was measured in a Microplate Spectrophotometer at a wavelength of 490 nm. The blank was subtracted to the measured optical density (OD) values, and the cell viability was normalized to the absorbance measured from the untreated control cells.

7. The loading of pyrene into BSA-PHPMA nanoparticles.

The BSA-PHPMA nanoparticles were synthesized using 5 % (w/w) solids content

as mentioned above. Before initiating the polymerization, 10 μL of pyrene THF solution (2 mg/mL) was added to the mixture. Then after the solutions being deoxygenated for 10 min, the reaction vessels were irradiated under blue LED light at room temperature. The reaction was quenched via turning off the blue light at predetermined time. The fluorescence emission spectra of all samples were recorded on a LS-55 fluorescence spectrometer (Perkin Elmer Co.) at 335 nm excitation wavelength.

8. The loading of Nile Red into BSA-PHPMA nanoparticles.

The BSA-PHPMA nanoparticles were synthesized using 5% (w/w) solids content as mentioned above. Before initiating the polymerization, 5 μL of Nile Red THF solution (2 mg/mL) was added to the mixture. Then after the solutions being deoxygenated for 10 min, the reaction vessels were irradiated under blue LED light at room temperature. The reaction was quenched via turning off the blue light after 360 min. The fluorescence of the samples were determined with a LS-55 fluorescence spectrometer (Perkin Elmer Co.) (ex. 365nm).

9. Loading and release behavior of doxorubicin hydrochloride (DOX)/DNA-SYBR Green I.

The preparation of BSA-PHPMA nanoparticles at 5% (w/w) solids content was described above. Before initiating the polymerization, 10 μL of DOX water solution (1 mg/mL) or 50 μL of DNA-SYBR Green I water solution (5 mg/mL) was added to the mixture. Then after the solutions being deoxygenated for 10 min, the reaction vessels were irradiated under blue LED light at room temperature for 360 min. The nanoparticles were purified by centrifuging to remove any unreacted monomer and free encapsulants. Then adding different amount of protease into the solution, 0.5 mg, 1.0 mg and 1.5 mg, respectively, the release of the loaded substances was monitored by fluorescence spectra (DOX: ex. 475 nm, em. 585 nm, and DNA-SYBR Green I: ex. 497 nm, em. 520nm) and the reaction vessels were placed into a water bath at 25°C.

10. Study loading efficiency of Doxorubicin and DNA-SYBR Green I in the nanoparticles.

10.1 Determination of loading efficiency of Doxorubicin (DOX)

The DOX content of BSA-PHPMA nanoparticles was determined by measuring the fluorescence of the nanoparticles solution in comparison to DOX in water. The DOX

content of the BSA-PHPMA nanoparticles solution was 1.35×10^{-3} mg/mL (Figure S20). Then, loading efficiency of DOX in BSA-PHPMA nanoparticles was calculated by equation S1. The DOX feed ($c_{DOX,feed}$) is 0.01 mg/mL. The amount of entrapped DOX ($c_{DOX,\ Entrapped}$) is 1.35×10^{-3} mg/mL. As a result, the entrapment efficiency of DOX is 13.5%.

$$\text{Loading efficiency} = c_{Entrapped} / c_{feed} \times 100\% \qquad \text{(eq. S1)}$$

10.2 Determination of DNA-SYBR Green I Content.

The DNA-SYBR Green I content of the BSA-PHPMA nanoparticles was determined by measuring the fluorescence of the BSA-PHPMA nanoparticles solution in comparison to DNA-SYBR Green I in water. The DNA-SYBR Green I content of the BSA-PHPMA nanoparticle solution was 29×10^{-3} mg/mL (Figure S21). Then, the loading efficiency of DNA-SYBR Green I in BSA-PHPMA nanoparticles was calculated by equation S1. The DNA-SYBR Green I feed ($c_{DNA,feed}$) was 0.25 mg/mL. The amount of entrapped DNA-SYBR Green I ($c_{DNA,\ Entrapped}$) was 29×10^{-3} mg/mL. As a result, the entrapment efficiency of DNA-SYBR Green I was 11.6%.

Characterization Methods

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy.

The mercaptothiazoline-activated trithiol-RAFT agent 4-cyano-4-(propyl-sulfanyl thiocarbonyl) sulfanylpentanoic acid mercaptothiazoline amide was dissolved in deuterated $CDCl_3$. PHPMA was dissolved in deuterated DMSO-d_6 which was obtained from the free BSA-PHPMA conjugates in aqueous solution. The ^{1}H NMR spectra were recorded on a JEOL ECX-400 400 MHz spectrometer.

UV−vis spectroscopy.

UV−vis spectroscopy studies were conducted using a Lambda 750S UV−vis spectrophotometer (Perkin Elmer Co.).

Fluorescence Spectroscopy.

The fluorescence of the samples were determined on a LS-55 fluorescence spectrometer (Perkin Elmer Co.).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was conducted at 120V using Tris-Glycine SDS (pH 8.3) as running buffer. And we used a 5% stacking gel and 10% separation gel. The samples were loaded by mixing 10 μL of measured solution (1 mg/mL) with 10 μL of 2× loading buffer, followed by Coomassie Blue staining.

Dynamic light scattering (DLS).

DLS measurements were operated at 25 °C using a scattering angle of 90° and at a laser wavelength of 633 nm with a Malvern Zetasizer Nano ZSP series instrument (Malvern Co.). In each case, the concentration of the sample was 1 mg/mL (protein).

Matrix-assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS).

MALDI-TOF MS was performed on a 4700 Proteomics analyser (Applied Biosystems) using 2,5-dihydroxybenzoic acid as matrix substance with a MALDI micro MX(Waters Co.). The sample aqueous solution concentration was 10 mg/mL.

Gel permeation chromatography (GPC).

To characterize the molecular weight of grafted PHPMA, we cleaved the polymer from BSA by hydrolysis. In each case, 1 mg of protease was added to the polymerization solution and the reaction vessels were immersed into a water bath at 25 °C for 24 hours. The grafted PHPMA product was obtained by centrifuging and then freeze-drying. The molecular weights and polydispersities were performed using a system equipped with a Hitachi L-2130 HPLC pump, a Hitachi L-2350 column oven operated at 40 °C, three Varian PL columns with 1,000K-100K (100,000A), 100K-10K(10,000A), and 100K-10K (1,000A) molecular ranges, and a Hitachi L-2490 refractive index detector. The GPC eluent was HPLC grade DMF at a flow rate of 1.0 mL/min. The apparent molecular weight was calculated based on PMMA standards.

Transmission Electron Microscopy (TEM).

TEM images were acquired using a JEM-1400 instrument (JEOL Co.) operating under UHV at 120 kV. To prepare samples, 2 μL of a dilute BSA-PHPMA nanoparticles solution was added onto a 300-mesh a carbon-coated copper grid for 30 s and then blotted with filter paper to remove excess solution. Finally, the samples were dried in vacuum for 2 days.

Scanning electron microscopy (SEM).

The images of the generated nanoparticles were studied by SEM using a FE-SEM SU8000 instrument (Hitachi Co.). Samples were prepared by adding 2 μL of a dilute BSA-PHPMA nanoparticles solution onto a wafer for 30 s and then removed excess solution with filter paper.

Supplementary Figures

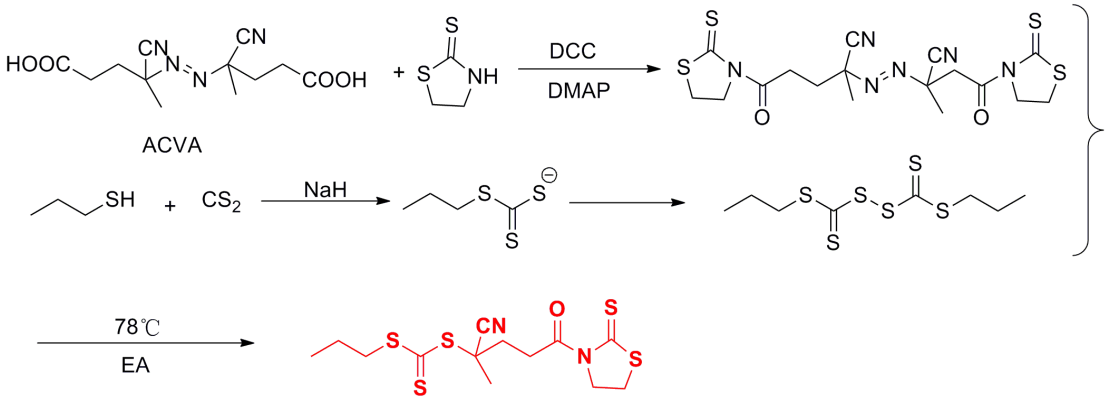

Figure S1. The general procedure for the synthesis of the mercaptothiazoline activated RAFT agent.

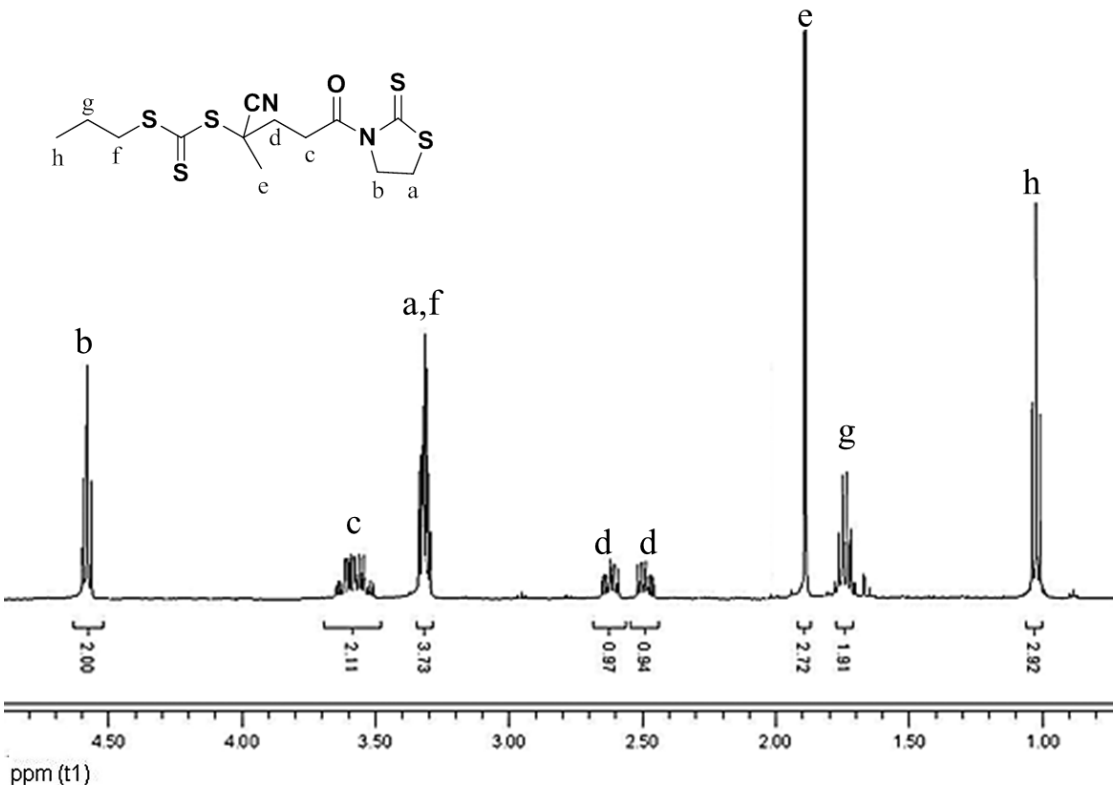

Figure S2. ^{1}H NMR spectrum of mercaptothiazoline-activated trithiol-RAFT agent 4-cyano-4-(propyllsulfanylthiocarbonyl) sulfanylpentanoic acid mercaptothiazoline amide in $CDCl_3$.

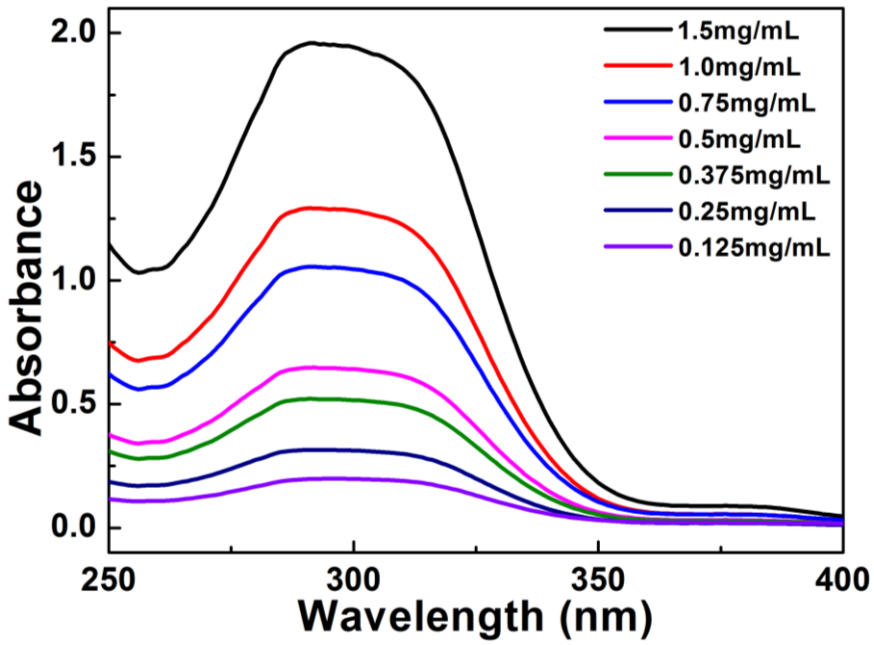

Figure S3. UV-Vis spectra of BSA-CTA_8 under different concentration (1.5mg/mL, 1.0 mg/mL, 0.75 mg/mL, 0.5 mg/mL, 0.375 mg/mL, 0.25 mg/mL and 0.125 mg/mL, respectively).

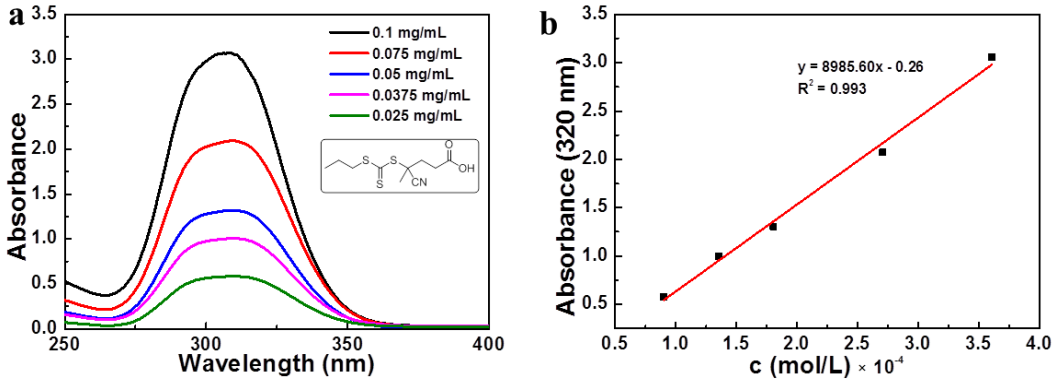

Figure S4. (a) UV-Vis spectra of a trithiol-RAFT agent under different concentration from 0.025 to 0.1 mg/mL, and (b) corresponding calibration curve based on plotting the absorbance at 320 nm against the concentration of trithiocarbonate.

Table S1. The composition of the BSA-CTA.

(nBSA : nRAFT) $_{\text{theoretical}}$	**(nBSA : nRAFT)** $_{\text{experimental}}$
1 : 5	1 : 5
1 : 10	1 : 9

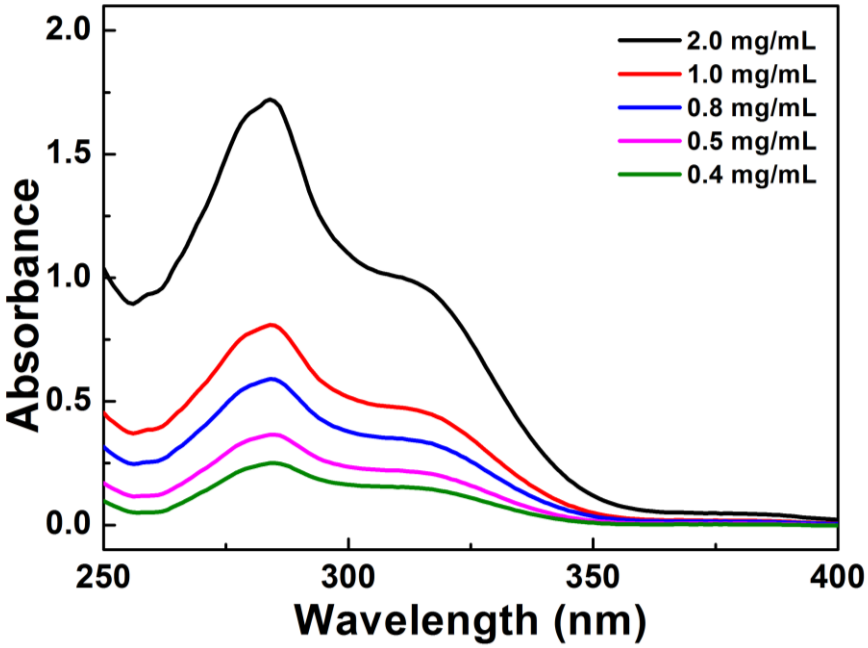

Figure S5. UV-Vis spectra obtained for BSA-CTA_5 under different concentration conditions (2.0mg/mL, 1.0 mg/mL, 0.8 mg/mL, 0.5 mg/mL and 0.4 mg/mL, respectively).

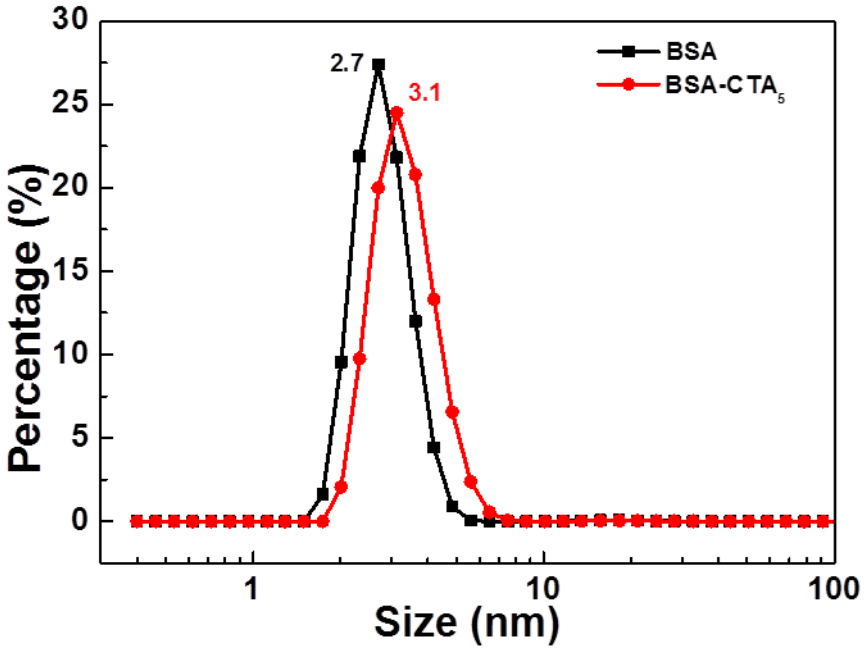

Figure S6. DLS of native BSA (black line) and BSA-CTA_5 (red line), showing an obvious hydrodynamic diameter increase for the BSA-CTA_5 compared with that of native BSA.

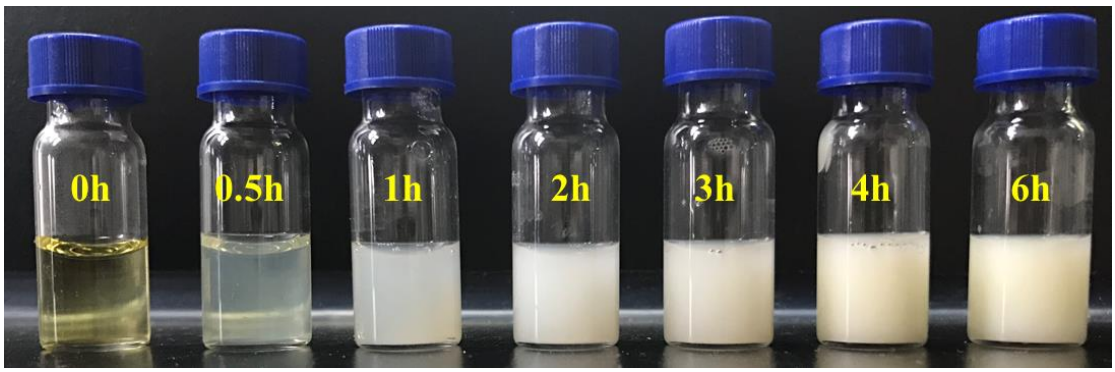

Figure S7. The photo images of the solution under different polymerization time using a BSA-CTA_8 as initiator under 5 % (w/w) solids content showed the change of the turbility of the solutions from transparent to opaque.

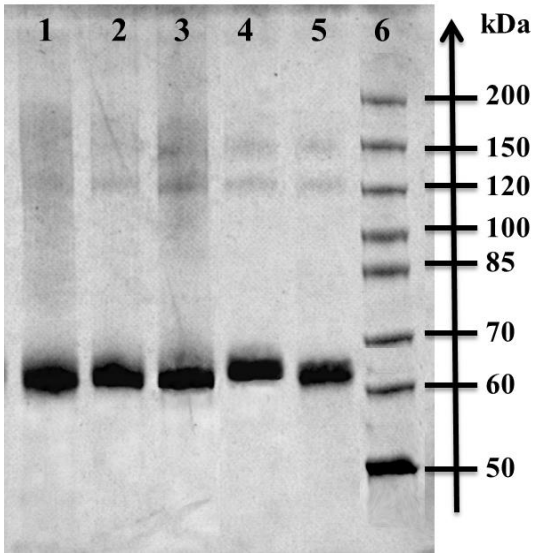

Figure S8. (a) SDS-PAGE of unpurified BSA-PHPMA conjugates using a BSA-CTA_8 as macro-RAFT agent under 5 % (w/w) solids content, lane 1: BSA-PHPMA conjugates after polymerization for 6 h, lane 2: BSA-PHPMA conjugates after polymerization for 2 h, lane 3: BSA-PHPMA conjugates after polymerization for 1 h, lane 4: BSA-CTA_8, lane 5: BSA, lane 6: protein marker. The bands at 67 kDa in lane 1-3 were from the unreacted BSA in the system which were much easier to be discerned in the SDS-PAGE.

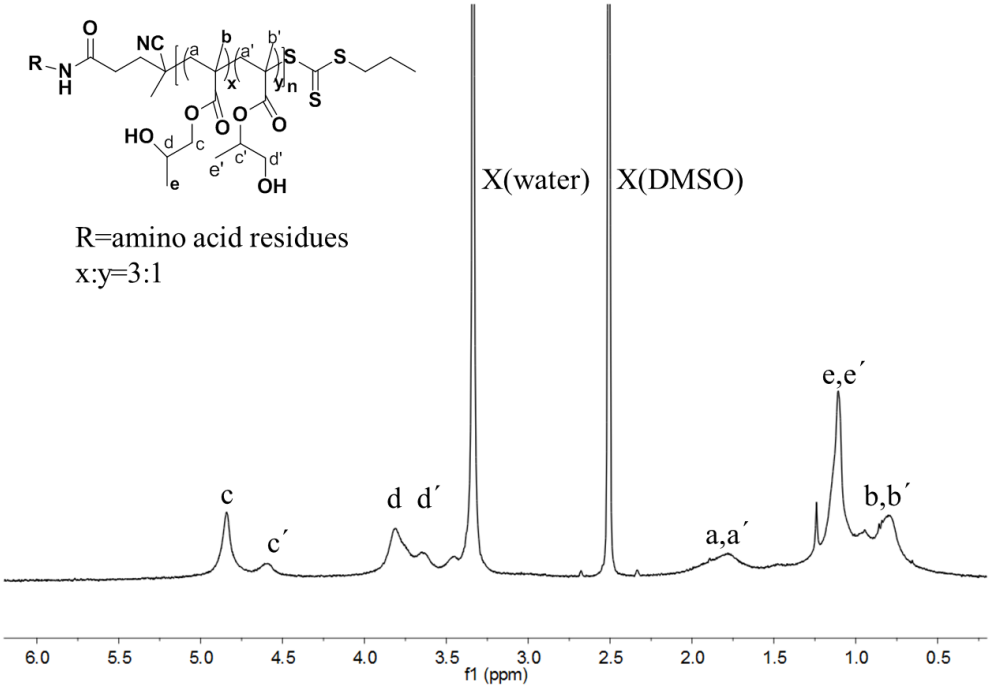

Figure S9. ^{1}H NMR spectrum of PHPMA in DMSO-d_6. PHPMA was obtained by hydrolyzing the free BSA-PHPMA conjugates with protease. The inset shows the chemical structure of PHPMA with full peak identification of PHPMA isomers. The HPMA monomer is actually an isomeric mixture composed of 75% of 2-hydroxypropyl methacrylate and 25% of 2-hydroxyisopropyl methacrylate.[8,9]

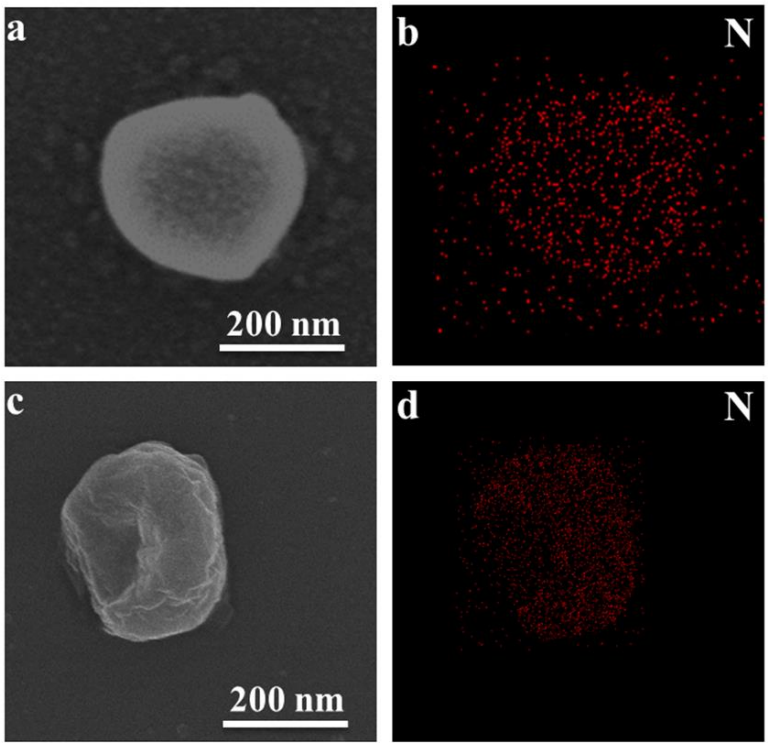

Figure S10. (a) SEM image of single BSA-PHPMA nanoparticle after polymerization for 6 h under 5 % (w/w) solids content, and (b) the corresponding nitrogen elemental mapping analysis. (c) SEM image of single BSA-PHPMA nanoparticle after being treated by protease (1 mg/mL) for 60 min, and (d) the corresponding nitrogen elemental mapping analysis.

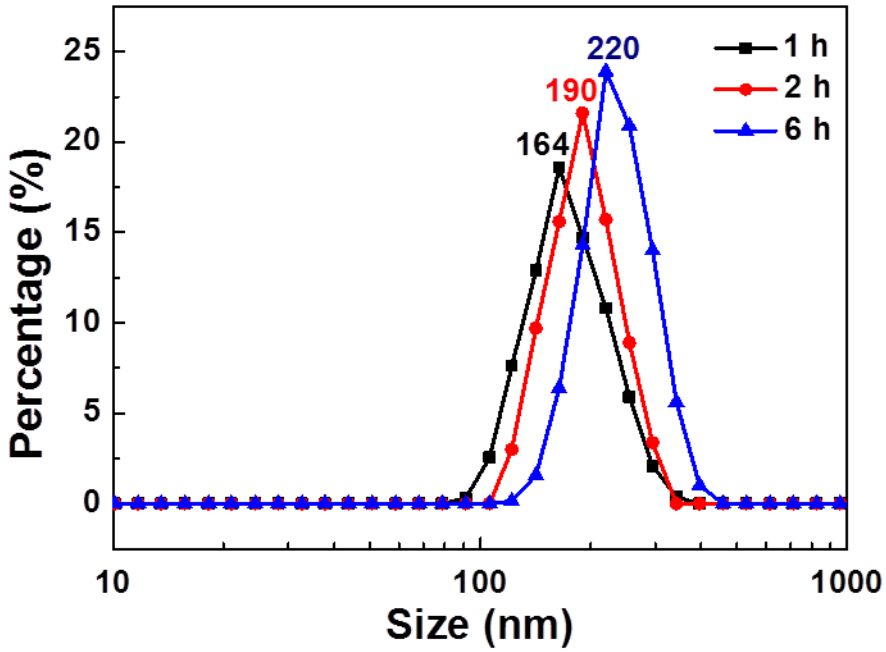

Figure S11. DLS data of BSA-PHPMA nanoparticles which polymerized for 1 h, 2 h and 6 h using a BSA-CTA_8 as macro-RAFT agent under 5 % (w/w) solids content showed hydrodynamic diameters ranging from 164 nm to 220 nm.

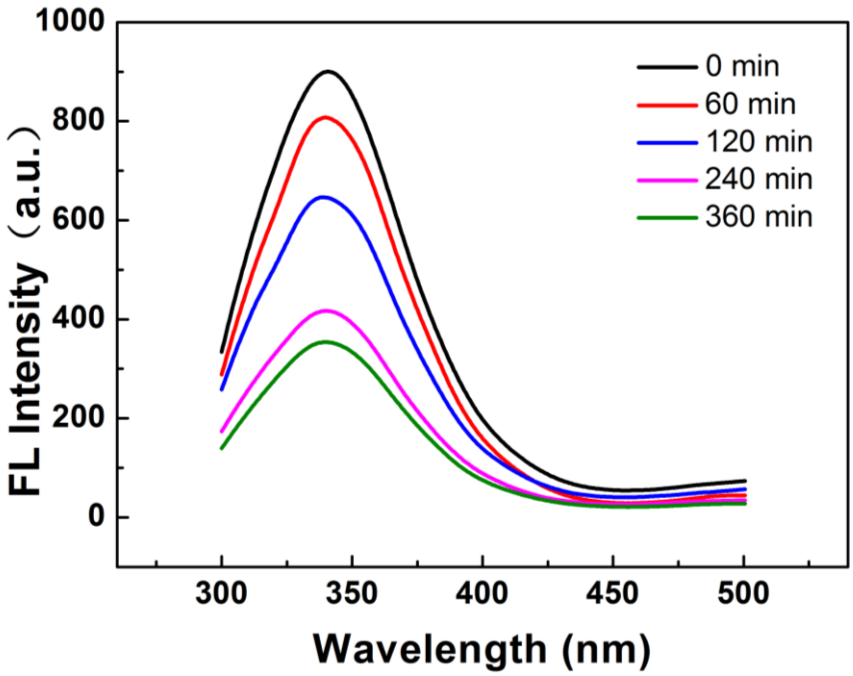

Figure S12. Fluorescence spectra was used to monitor the decrease of the concentration of BSA in the aqueous solution. Under different polymerization time, after centrifuging, the supernatant was measured directly.

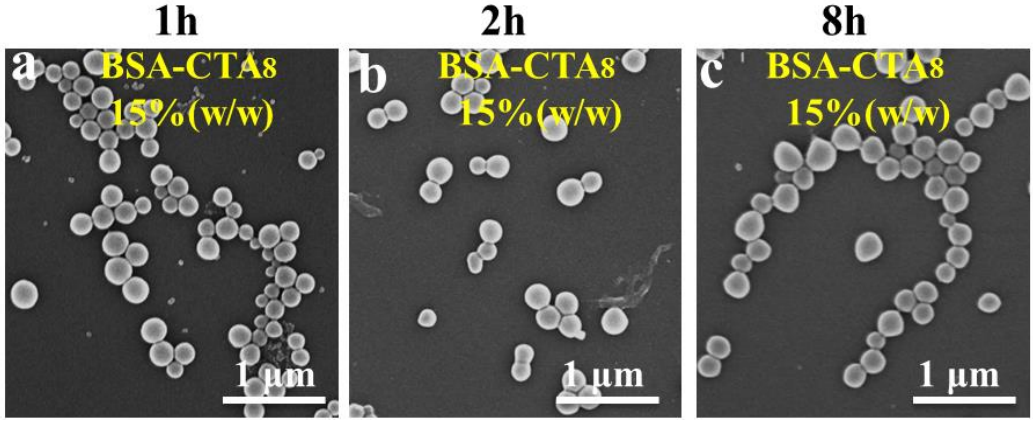

Figure S13. SEM images of the BSA-PHPMA nanoparticles morphologies obtained under different polymerization time using BSA-CTA_8 at 15% (w/w) solids content, which corresponded to (a) 1 h, (b) 2 h and (c) 8 h.

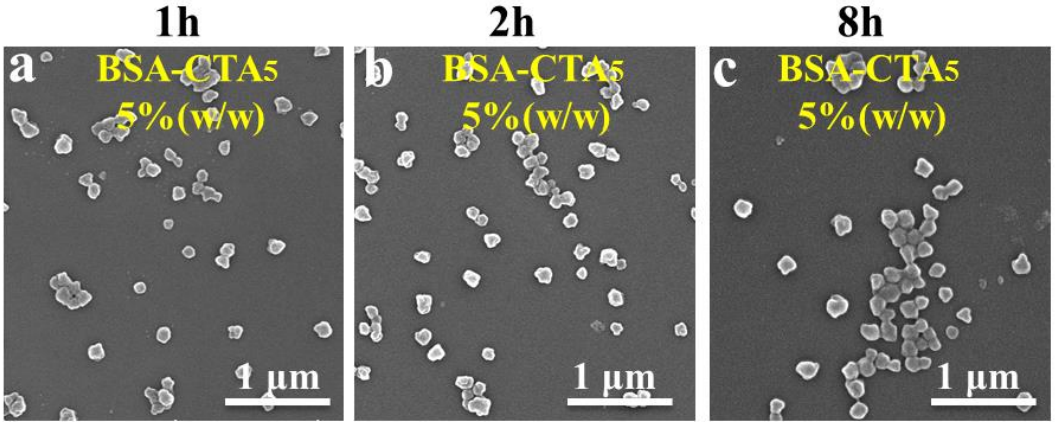

Figure S14. SEM images of the BSA-PHPMA nanoparticles morphologies obtained under different polymerization time using BSA-CTA_5 at 5% (w/w) solids content, which corresponded to (a) 1 h, (b) 2 h and (c) 8 h.

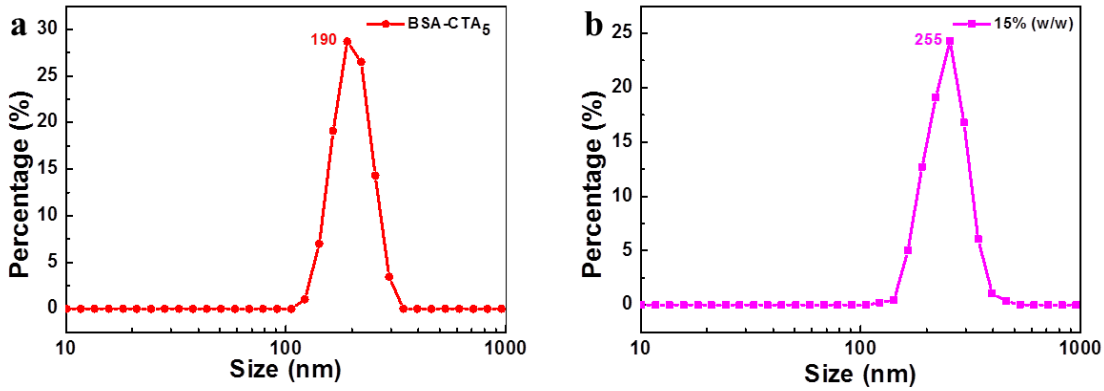

Figure S15. DLS data showed the diameter of BSA-PHPMA nanoparticles obtained by using (a) BSA-CTA_5 at 5 % (w/w) solids content, and (b) BSA-CTA_8 at 15 % (w/w) solids content.

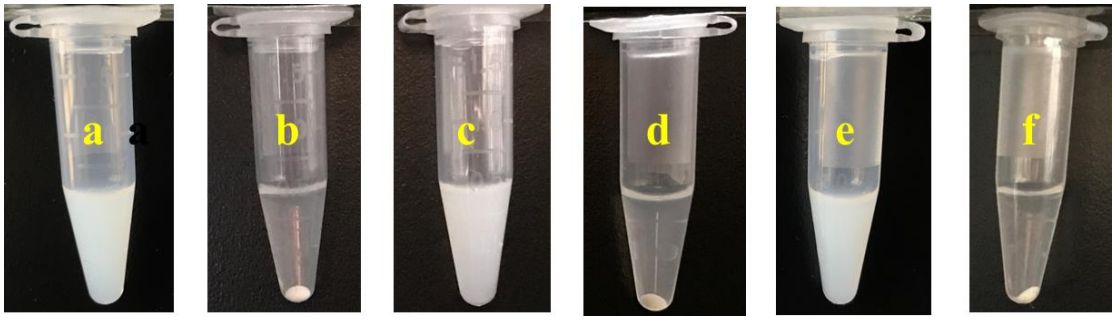

Figure S16. Photo images showed the continuous centrifugation-redispersion procedure of the BSA-PHPMA nanoparticles in aqueous solution. (a) before and (b) after centrifugation, (c, d) after one cycle, and (e, f) after two cycles.

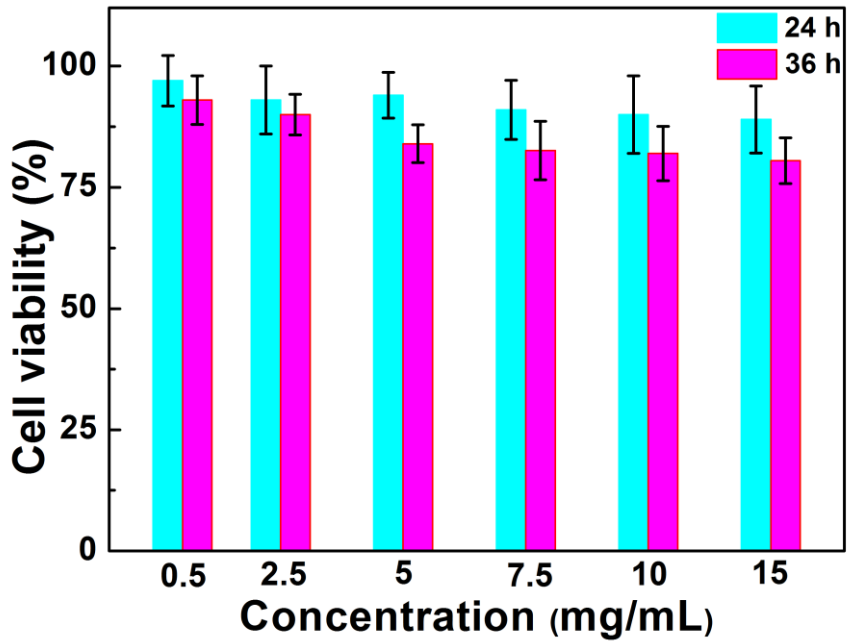

Figure S17. Cytotoxicity of BSA-PHPMA nanoparticles to normal NIH 3T3 cells after 24 h (blue bar) and 36 h (purple bar) under different concentration of BSA-PHPMA nanoparticles from 0.5 mg/mL to 15 mg/mL.

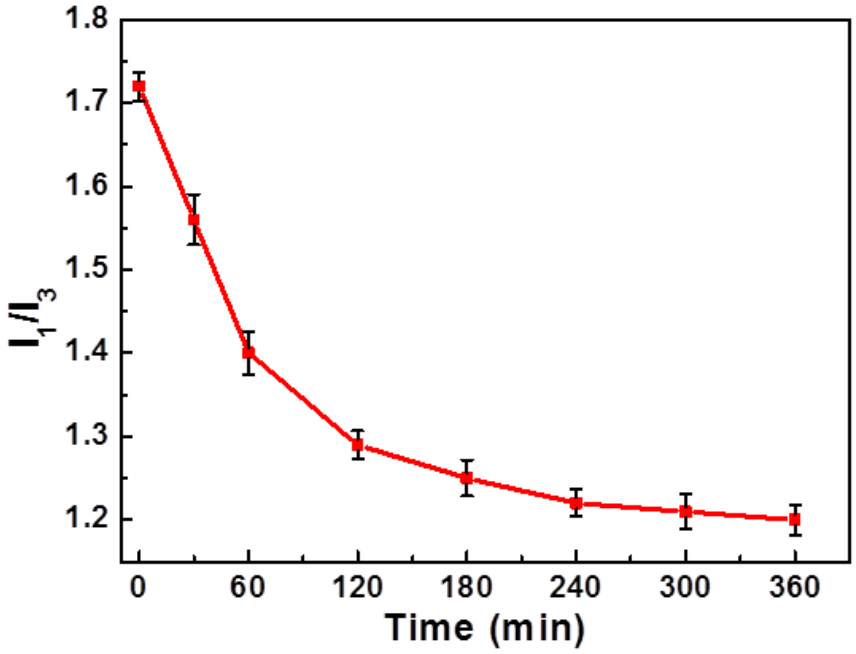

Figure S18. Relationship between the fluorescent intensity ratio (I_1/I_3) and polymerization time in water, where I_1 and I_3 were the emission intensities of the first and third bands in the fluorescence spectrum of pyrene, respectively.

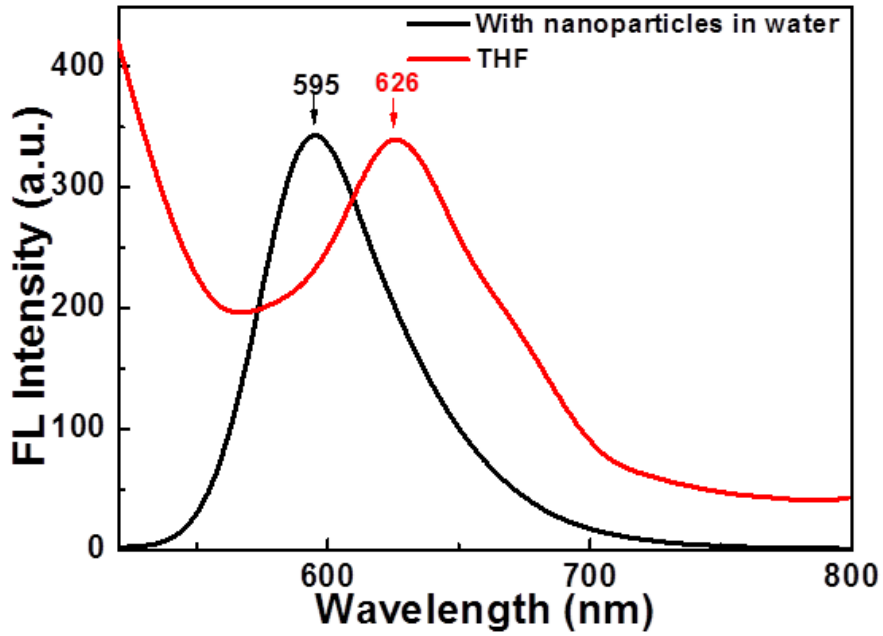

Figure S19. Fluorescence spectra of Nile Red in THF (red line) and inside BSA-PHPMA nanoparticles in water (black line), showing that Nile Red could be encapsulated inside the nanoparticles.

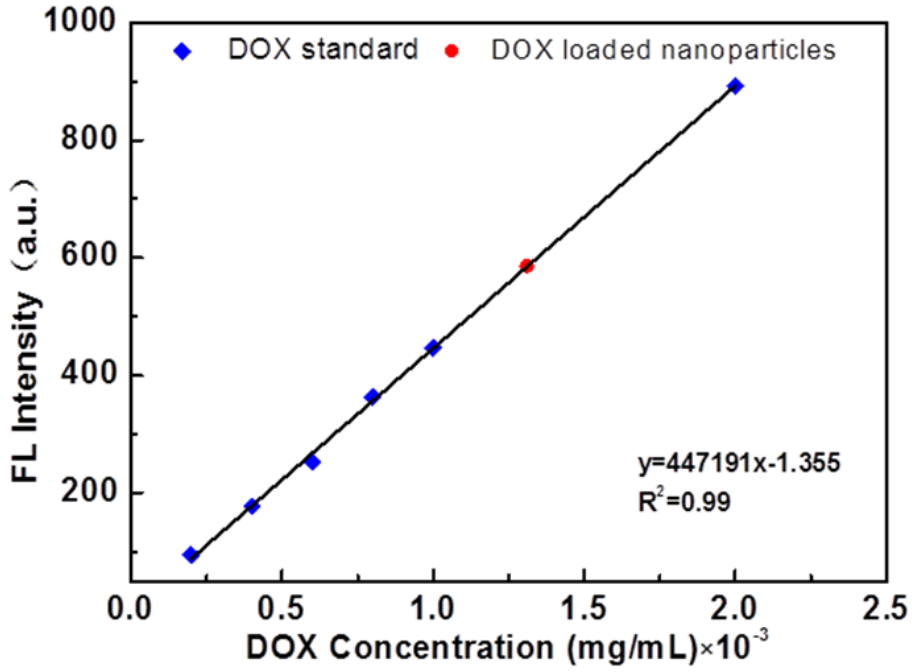

Figure S20. Determination of the DOX content of DOX-loaded BSA-PHPMA nanoparticles (●) by using a standard curve of DOX (◆).

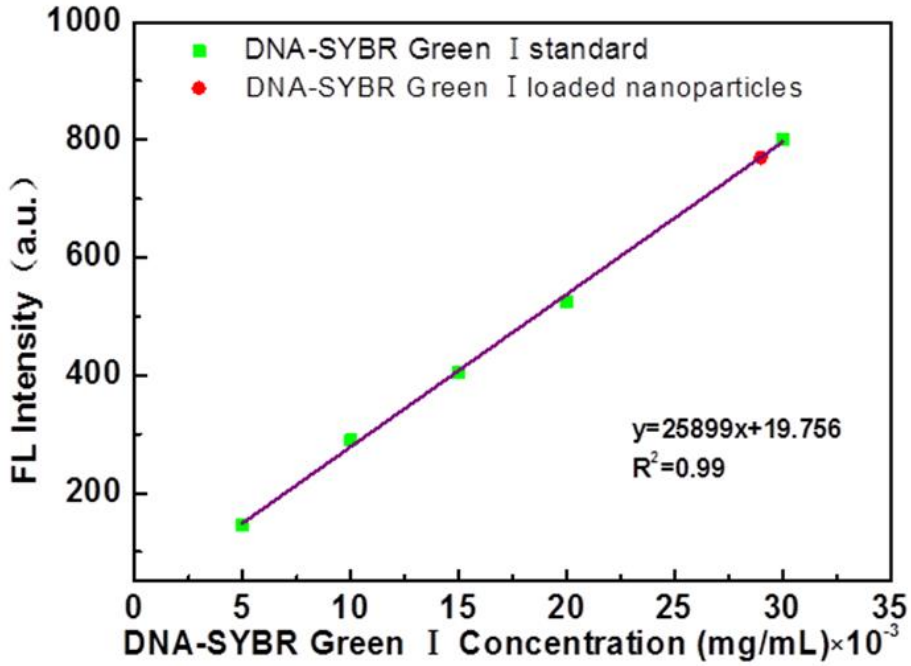

Figure S21. Determination of the DNA-SYBR Green I content of DNA-SYBR Green I -loaded BSA-PHPMA nanoparticles (●) by using a standard curve of DNA-SYBR Green I (■).

References

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