Electronic Supplementary Information (ESI)

Visible Light Induced Fast Synthesis of Protein-Polymer Conjugates: Controllable Polymerization and Protein Activity

Xin Li,[a,§] Lei Wang,[a,§] Gaojian Chen,[a,b] David M. Haddleton[c] and Hong Chen*[a]*

[a] *The Key Lab of Health Chemistry and Molecular Diagnosis of Suzhou, Chemical Engineering and Materials Science, Soochow University, 199 Ren-Ai Road, Suzhou, 215123, P. R. China*

[b] *Center for Soft Condensed Matter Physics and Interdisciplinary Research, Soochow University, Suzhou, 215006, P. R. China*

[c] *Department of Chemistry, University of Warwick, Coventry, CV47AL, UK*

E-mail: chenh@suda.edu.cn; gchen@suda.edu.cn

Experimental Section

1. Materials

N-Isopropylacrylamide (NIPAm, Acros, 99%) was recrystallized from a toluene/hexane mixture (50% v/v) and dried under vacuumprior to use. N,N-Dimethylformamide (DMF, Aldrich, 99%), (2,4,6-trimethylbenzoyl) phenylphosphinate (TPO-L, TCI, 95%), 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, Aldrich, 98%), N,N'-dicyclohexylcarbodiimide (DCC, Aldrich, 99%), 4-dimethylaminopyridine (DMAP, Aldrich, 99%), chloroform-d ($CDCl_3$, Aldrich, 99.96%), and deuterium oxide (D_2O, Aldrich, 99.9%) were used as received. Dialysis membranes (molecular weight cut-off (MWCO): 8000-14,000 Da) were obtained from Solarbio Science & Technology Co., Ltd. The centrifuge filters (Amicon Ultra-15, MWCO 10,000 Da) were purchased from Millipore Corporation. Deionized water (DIW) was purified using a Millipore water purification system to give a minimum resistivity of 18.2 MΩ•cm. All other chemicals were purchased from Shanghai

Chemical Reagent Co. and used without further purification, unless otherwise specified.

2. Analytical Techniques

Aqueous Gel Permeation Chromatography (GPC). Aqueous GPC was performed on a Agilent 1200 HPLC system equipped with a UV detector and a Sepax Zenix SEC-300column (7.8 x 300 mm column, pore size 300 Å, particle size 3 μm) using a 150 mM phosphate buffer at pH = 7.0 as the mobile phase at 15 °C (flow rate: 0.4 mL/min). Chromatograms were acquired at 215 nm wavelength.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was carried out using a 4% stacking and a 10% separating gel in a Mini-Protein II apparatus (Bio-Rad). Samples were prepared at 1 mg/mL concentration in Tris buffer containing SDS, bromophenol blue and glycerol with addition of 2% β-mercaptoethanol for reducing conditions. The gels were stained with Coomassie Brilliant Blue and examined with an EC3 imaging system.

Mass Analysis. Liquid chromatography-mass spectrometry **(**LC-MS) analyses were performed on Agilent 6220 Quadrupole LC/MS system with an ESI resource.

Matrix-Assisted Laser Desorption Ionization Spectroscopy (MALDI-TOF). MALDI-TOF spectra were collected using a Bruker Daltonics Ultraflextreme mass spectrometer equipped with a Nd:YAG laser (355 nm) in positive linear mode operated with flexControl 3.4 software.

UV-vis Spectroscopy. UV-vis spectra were recorded using a spectrophotometer (Varioskan Flash, Thermo Scientific, USA) equipped with a temperature controller.

Dynamic Light Scattering (DLS). DLS studies of the protein and conjugates at 0.5 mg/mL in an aqueous were conducted using a Zetasizer Nano-ZS90 (Malvern Instrument Ltd. UK) at designed temperature.

NMR Spectroscopy. ^{1}H NMR and ^{13}C NMR spectra were recorded on an INOVA 400 MHz nuclear magnetic resonance instrument, with D_2O or $CDCl_3$ used as solvents.

Circular Dichroism measurements (CD). The CD spectra were obtained on a

AVIV 410 Circular Dichroism Spectrometer (AVIV Biomedical, Inc. Lakewood, NJ USA) at room temperature with a 0.2 cm quartz cuvette. The protein samples were dissolved in DIW at a protein concentration of 0.08 mg/mL measured from 190 nm to 250 nm and a concentration of 2 mg/mL measured from 250 nm to 350 nm. The absorbance of the enzyme solution at each wavelength was maintained below 1.2. Each CD spectrum was the average of three scans performed at a scan rate of 20 nm/min. Background scans without protein were also obtained and subtracted from the wavelength scans prior to converting the millidegrees to the mean residue molar ellipticity.

3. Methods and Results

3.1. Mutation and Expression of PPase.

3.1.a Bacterial Strains, Plasmids and Culture Conditions.

The following strains of *E. coli* were used: K-12 is a wild-type strain from this laboratory and XLI-Blue was used as the host for gene cloning and protein expression. The plasmid pQE30 was used as the vector for production of the native and mutant PPases. The strains were grown in either liquid LB medium (Luria nutrient medium consisting of 1% Bactotryptone, 0.5% NaCl, and 0.5% Bacto yeast extract, pH 7.0) or on LB plates (Luria nutrient medium containing 1.5% agar). Ampicillin (0.1 mg/mL) and tetracycline hydrochloride (0.025 mg/mL) were added when required.

3.1.b Cloning of the *ppa* Gene.

Polymerase chain reaction (PCR) was used to clone the *ppa* gene encoding the *E. coli* PPase. The chromosomal DNA isolated from *E. coli* K-12 was used as the PCR template, and the primers used for the reaction were 5'-CGCGGATCCAGCTTACTCAACGTCCCT-3' and 5'-CGCAAGCTTTTATTTATTCTTTGCGCGCTC-3'. The PCR was performed in a thermal cycler (MasterCycler ep gradient S, Eppendorf, Germany). The PCR product was digested with BamHI and HindIII. The digest was fractionated by gel electrophoresis in an agarose gel, and a DNA fragment of approximately 0.5 kb was

extracted from the gel with a gel extraction kit (Axygen). This fragment was ligated into the BamHI-HindIII site of the pQE30 vector. The plasmids were transformed into *E. coli* XLI-Blue, and the transformants containing the *ppa* gene were screened for the production of *E. coli* PPase.

3.1.c **Site-directed Mutagenesis.**

Site-directed mutagenesis was performed according to the megaprimer PCR method using Pfu DNA polymerase.[1] The forward flanking primer sequence used in this experiment was 5'-CGCAAGCTTTTATTTATTCTTTGCGCGCTC-3', and the reverse flanking primer sequence was 5'-CGCGGATCCAGCTTACTCAACGTCCCT-3'. The mutagenic primer used to create the K148C-PPase was 5'- CCTCGAAAAAGGCTGCTGGGTGAAAGTTGAAGG-3' (nucleotides that represent mutations are underlined). The megaprimer PCR products were then digested with BamHI and HindIII and ligated into the BamHI-HindIII site of the pQE30 vector as described previously. DNA sequencing was performed by Sangon Biotech (Shanghai) Co., Ltd.

3.1.d **Expression and Purification.**

For the expression of PPase, strains containing the recombinant plasmids encoding mutant PPase were grown in liquid LB medium overnight on a shaker at 37 ^{o}C. From the stationary overnight culture, a new culture was started in liquid LB medium at 1:100 dilution. This culture was incubated on a shaker at 37 ^{o}C until it reached an OD_{600} of 0.5. Isopropyl β-D-1-Thiogalactopyranoside (IPTG) (0.5 mM) was added to induce the expression of PPase, and the culture was incubated with shaking at 37 ^{o}C for 3 h. The cells were pelleted by centrifugation and disrupted with lysozyme and sonication in 50 mM phosphate buffer (pH 8.0). The cell debris was removed by centrifugation, and the PPase in the supernatant was purified over Ni-NTA Sepharose Resin (Shanghai Sangon Biotech Co., Ltd., China) according to the manufacturer's instructions. The enzymes were then purified and concentrated using centrifuge filters (Amicon Millipore with 50,000 Da MWCO). The purity of the enzyme preparations was verified by SDS-PAGE.

3.2. Synthesis of Water-soluble Photoinitiator (2,4,6-trimethylbenzoyl) PhenylPhosphonicAcidSodium,TPO-Na).

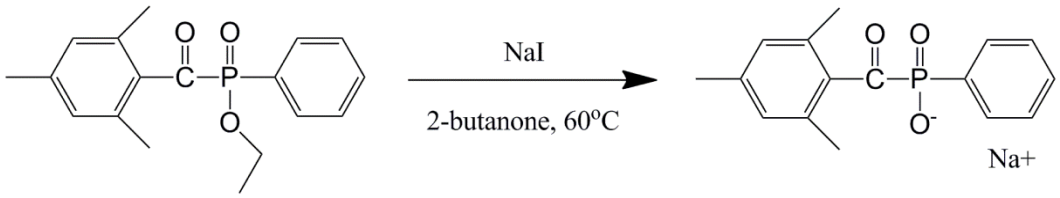

Fig. S1 Synthesis of TPO-Na.

TPO-L (3.16 g, 10 mmol) and sodium iodide (1.6 g, 11 mol) were dissolved in 2-butanone (30 mL), the reaction was carried out at 60 °C under nitrogen overnight. The precipitate was collected, washed with 2-butanone twice and then three times with diethyl ether. The final product was dried in the dark for 48 h under vacuum to give TPO-Na (1.38 g, 44% yield) as a white solid. ^{1}H-NMR (400 MHz, D_2O, 298 K): δ: 7.74-7.40 (m, 5H, Ar-***H***), 6.86 (s, 2H, Ar-***H*** **(**-C***H***=C-CH_3)), 2.21 (s, 3H, -CH=C-C***H***$_3$), 1.99 (s, 6H, -CO-C=C-C***H***$_3$). ^{13}C-NMR (400 MHz, D_2O, 298 K): δ: 228.6 (***C***=O), 138.6, 133.7, 132.3, 132.2, 132.1, 128.5, 128.4, 128.2 (***C***H of Ar), 20.2 (-CH-C-***C***H_3), 18.6 (-CO-C-C-***C***H_3). MS (m/z) calcd for $C_{16}H_{16}O_3PNa$: 309.3[M-H]$^-$; found: 309.4.

3.3. Synthesis of the Maleimide Chain Transfer Agent (Maleimide-CTA), Butylsulfanylthiocarbonylsulfanyl-2-methylpropionic Acid Maleimide Methyl ester.

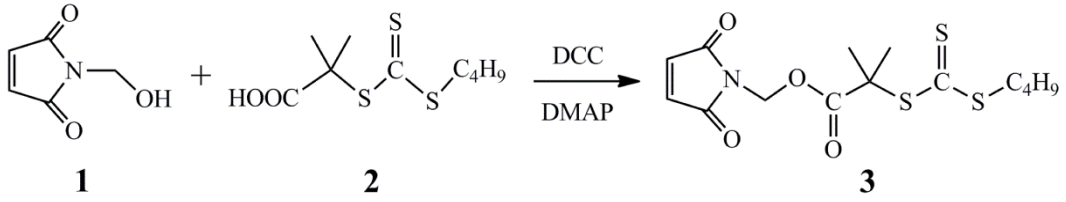

Fig. S2 Modification of CTA with maleimide group.

N-Methylolmaleimide 1 (330.5 mg, 2.6 mmol) and CTA 2 (605.8 mg, 2.4 mmol) were synthesized according to literatures[2] and added to a round-bottom flask with DMAP (29 mg, 0.24 mmol), dissolved in dichloromethane anhydrous (DCM, 15 mL). The mixture was stirred at ice bath to cool down the system and purged with N_2 for 30 min. A solution of DCC (0.59 g, 2.85 mmol) in DCM (5 mL) was added slowly and kept in N_2 atmosphere at ice bath for 2 h and continued at room temperature overnight. The resulting solution was filtered and the solvent was evaporated under vacuum. The crude product was isolated by column chromatography (silica gel) using hexane: ethyl acetate (3: 1 v/v) as an eluent. The product was purified by recrystallization from hexanes and dried under vacuum to get yellow crystals (236 mg, 27% yield). ^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ: 6.79 (s, 2H, -C***H***=C***H***-CO), 5.57 (s, 2H, -N-C***H***$_2$-O-), 3.28 (m, 2H, S=C-S-C***H***$_2$-CH2-), 1.67 (s, 6H, (C***H***$_3$)$_2$-C-S-), 1.63 (m, 2H, –CH_2–C***H***$_2$–CH_2–CH_3),1.41 (m, 2H, –CH_2–CH_2–C***H***$_2$–CH_3), 0.91-0.88 (t, 3H, *J* = 14.8 Hz,–CH_2–CH_2–CH_2–C***H***$_3$). ^{13}C-NMR (400 MHz, $CDCl_3$, 298 K): δ: 221.2 (***C***=S), 171.9 (-CH_2-O-***C***=O), 168.7 (-CH=CH-***C***O), 134.7 (-*C*H=***C***H-CO), 61.2 (-N-***C***H_2-O), 55.5 (-S-***C***$(CH_3)_2$-CO), 36.7 (CH_3–CH_2–CH_2–***C***H_2–S), 29.9 (CH_3–CH_2–***C***H_2–CH_2–S), 25.2 (-C(***C***$H_3)_2$), 22.1 (CH_3–***C***H_2–CH_2–CH_2–S), 13.6 (***C***H_3–CH_2–CH_2–CH_2–S). MS (m/z) calcd for $C_{14}H_{19}S_3NO_4$: 362.5 $[M+H]^+$; found: 362.2.

3.4. Synthesis of PPase MacroRAFT Agent.

PPase (44 mg, 2.1 μmol) was dissolved in degassed phosphate buffer (PB) solution (21 mL, 0.1 M, pH 7.2) at a low stirring rate. A solution of maleimide-CTA (23 mg, 63 μmol) in nitrogen-purged DMF (1.4 mL) was added dropwise under nitrogen and the solution was stirred at ambient temperature for 4 h. The solution was centrifuged at 4 ^{o}C several times to remove excess maleimide-CTA. Subsequently, the supernatant was dialyzed against PB solution and water for 36 h using a MWCO of 8000-14,000 Da to eliminate the excess of the maleimide-CTA. The PPase macroRAFT agent was isolated by lyophilization and Ellman's assay (see below) proved almost complete disappearance of the thiol groups of PPase, which means the successful conjugation of PPase with maleimide-CTA.

3.4.a Determination of Free Thiols by Ellman'sAssay.

Free thiol content was determined using Ellman's assay.[3] Ellman's reagent (4.0 mg, 0.010 mmol) was dissolved in 1 mL sodium phosphate buffer solution (PB, pH 8.0, 0.1 M) containing 1 mM EDTA to prepare the Ellman's reagent solution. A 250 µL aliquot of the protein sample (at least 5.0 mg/mL), 50 µL Ellman's reagent solution and 2.50 mL PB were mixed for 20 min at room temperature. The absorbance at 412 nm was measured with a spectrophotometer. The thiol concentration was calculated using Beer-Lambert's law, with a molar extinction coefficient for 2-nitro-5-thiobenzoic acid=14,150 M^{-1} cm^{-1} at 412 nm (Fig. S3).

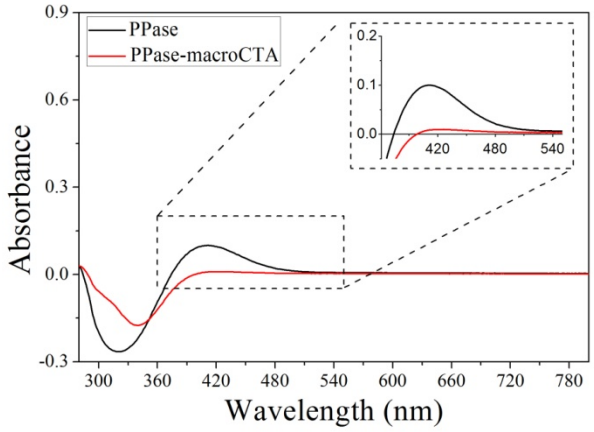

Fig. S3 Ellman's assay results of PPase before (black) and after (red) modification with maleimide-CTA.

3.4.b MALDI-TOF characterization of PPase-MacroCTA.

MALDI-TOF was employed to confirm the functionalization of PPase with the RAFT agent moiety. Protein solutions (0.8 mg/mL) were prepared by dissolving in acetonitrile/water (3:7 v/v) with 0.1% trifluoroacetic acid. The matrix, sinapic acid, was dissolved over-saturated in the same solvent system. Equal volumes of matrix and analyte were mixed and an aliquot (2 µL) of the resulting mixture spotted onto a plate target and allowed to dry. Each spectrum consists of at least 2000 average scans. The molar mass of PPase and PPase-macroCTA were calculated to be 20950 and 21376 g/mol. The MALDI-TOF spectra are provided in Fig. S4.

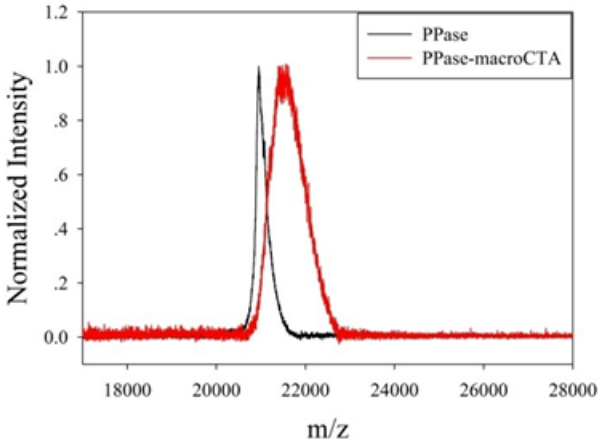

Fig. S4 MALDI-TOF spectra for PPase and PPase-macroCTA.

3.5. Aqueous Visible Light induced RAFT from PPase-MacroCTA.

PPase-macroCTA (42 mg, 2 μmol) and NIPAm monomer (113 mg, 1 mmol) were added into a glass round-bottom flask with magnetic stirring bar and degassed with Ar for 30 min. Then degassed DIW (3.3 mL) containing photoinitiator TPO-Na (3.1 mg, 1 μmol) was injected into the flask under dark condition, purified with Ar for another 30 min to make sure no oxygen in the flask. UV light was filtered by filter and polymerization was carried out at 20 ^{o}C under visible light radiation. Samples with different polymerization time were removed by syringe for characterization.

3.6 ^{1}H NMR measurement of the PPase-PNIPAm conjugates.

The degree of polymerization was obtained from the ^{1}H NMR spectra by comparing the integrations resulting from the methyne proton in the isopropyl -CH-$(CH_3)_2$ area of the PNIPAm side chains (chemical shift of 3.8 ppm, labeled with e in Fig. S5) with the methylene protons adjacent to the trithiocarbonate group (chemical shift of 2.7 ppm, labeled with g in Fig. S5).

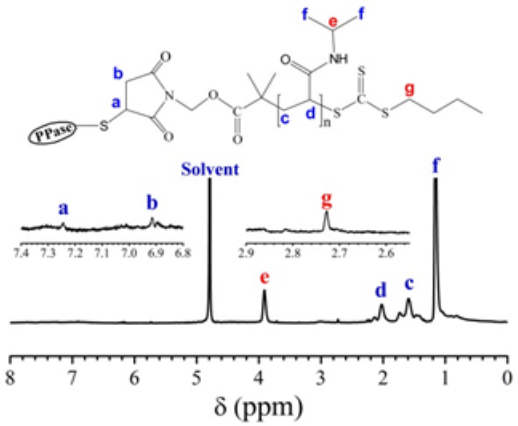

Fig. S5 Typical ^{1}H NMR spectrum of PPase-PNIPAm conjugate.

3.7. ^{1}H NMR and GPC measurements of Aqueous Visible Light induced RAFT by maleimide-CTA.

Maleimide-CTA (2.2 mg/mL dissolved in DMSO, 1 mL, 6 μmol) and NIPAm monomer (339.4 mg, 3mmol) were added into a glass round-bottom flask, and then degassed DIW (9 mL) containing photoinitiator TPO-Na (9.3 mg, 3 μmol) was injected into the flask under dark condition, purified with Ar for 1 h to make sure no oxygen in the flask. UV light was filtered by filter and polymerization was carried out at 20 ^{o}C under visible light radiation. Samples with different polymerization time were taken by syringe for ^{1}H NMR and GPC characterization (Fig. S6).

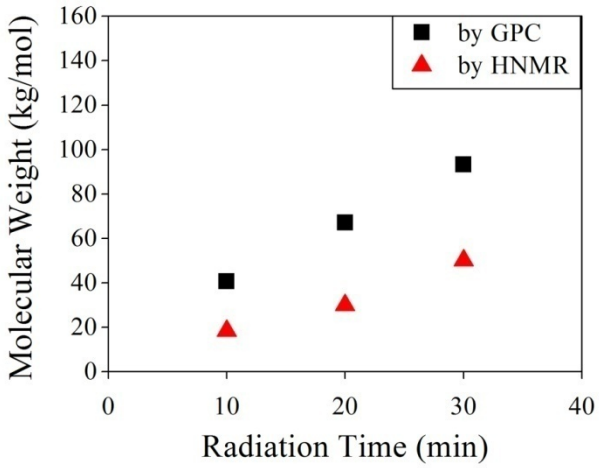

Fig. S6 Polymer molecular weight from maleimide-CTA by GPC (black) and ^{1}H NMR (red).

3.8. LCST Measurements.

PPase-PNIPAm conjugates and PNIPAm were respectively dissolved in DIW at a concentration of 1 mg/mL overnight to ensure complete dissolution. The solutions were warmed from 25 °C to 45 °C at an incubation rate of 3 min/°C and determined at each temperature degree by a UV-vis spectrophotometer at 500 nm. The LCST was determined to be the temperature at 10% of the maximum absorbance of the solution. The results are given in Fig. S7.

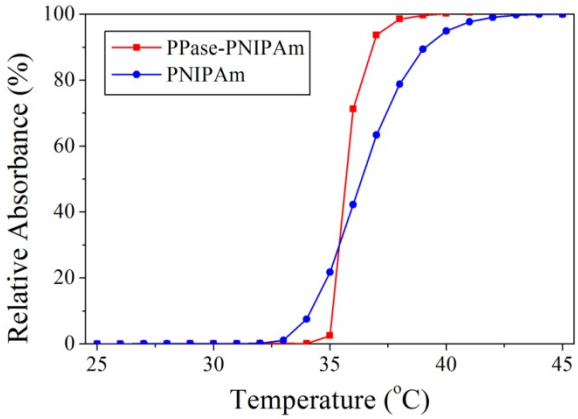

Fig. S7 LCST measurement of PPase-PNIPAm conjugates (red) and PNIPAm (blue) at 1 mg/mL solution in DIW.

3.9. DLS measurements.

DLS was used to characterize the distribution of PPase and conjugates blow and above the LCST. Samples were dissolved in water at a concentration of 0.5 mg/mL and cooled overnight to ensure complete dissolution. Before determination, the solutionswere filtered through a 0.2 μm syringe filter and then incubated at 25 °C for at least 1 h. Results in Fig. S8 shows that compared to PPase, hydrodynamic diameter of the PPase-PNIPAm conjugates at 25 °C increased from 7 nm to ~40 nm, which was due to the conjugation of polymer. For determination of the real state at 45 °C, solutions were then kept at 45 °C overnight. There's no appreciable change in size of PPase but an obvious increase to 150 nm because of intermolecular aggregation upon dehydration of the tethered PNIPAM chains.[3a, 4]

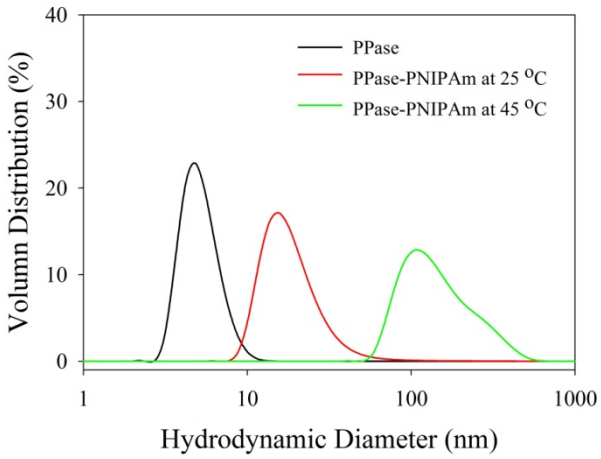

Fig. S8 Hydrodynamic diameter distributions of PPase-PNIPAm conjugates below and above the LCST.

3.10. Activity assay of PPase, PPase-macroCTA and PPase-PNIPAm conjugates.

The protein activity of the samples was assayed using the method of Heinonen and Lahti.[5] A PPase, PPase-macroCTA or PPase-PNIPAm conjugate solution (10 μL, PPase concentration: 5 μg/mL) in 50 mM Tris-HCl buffer (pH 8.0) was mixed with a solution of $MgCl_2$ (5 μL, 1 M) and Tris-HCl buffer (81 μL, 50 mM, pH 8.0). After incubating at designed temperature for 5 min, the solution was added into the substrate sodium pyrophosphate (4 μL, 50 mM) and kept at the corresponding temperature for another 10 min. The reaction (V=100 μL) was terminated by the addition of 10 μL 0.4 M citric acid, and then 800 μL daily acetone-acid-molybdate solution (AAM solution, acetone, 2.5 M sulfuric acid and 10 mM ammonium molybdate in 2: 1: 1 volume proportion) was added to the tubes. The contents were mixed carefully with a vortex mixer, and 80 μL citric acid (1 M) was pipetted into each tube. The mixed solution was measured at 355 nm. Measurements were performed with three different samples and reported as the average of 3 measurements ± standard deviation. Results are shown in Table S1. Enzyme activity is expressed in katal (kat=1 mol/s) per kg protein.

Table S1 Specific activity of proteins before and after modification.

Samples	Specific activity (kat/kg)		Ratio of activity
	at 25 °C	at 45 °C	(45 °C/25 °C)
PPase	3.35	7.07	2.1
PPase-macroCTA	0.38	0.84	2.2
PPase-PNIPAm10min	0.26	2.96	11.4
PPase-PNIPAm20min	0.31	3.45	11.1
PPase-PNIPAm30min	0.36	3.87	10.8

3.11. Control Experiment.

3.11.a Measurement of Activity of PPase under Visible Light Radiation.

PPase was dissolved in DIW and placed under visible light radiation with an intensity of 0.2 mW/cm^2 at 420 nm in 20 °C water bath as polymerization condition. Protein solution was extracted periodically for 10 min and activity measurement showed no difference in different radiation time samples, approving no influence of visible light on protein activity.

3.11.b Effcet of Free PNIPAm Chain on Protein Activity.

Activity of PPase and PPase with free PNIPAm (Mw=42272, PD=1.2, equal molar with protein) in different temperature were measured to show the effect of free PNIPAm chain on protein activity (Fig. S9). Results show that at the same molar amount free polymer has no impact on PPase activity, no matter below or above LCST.

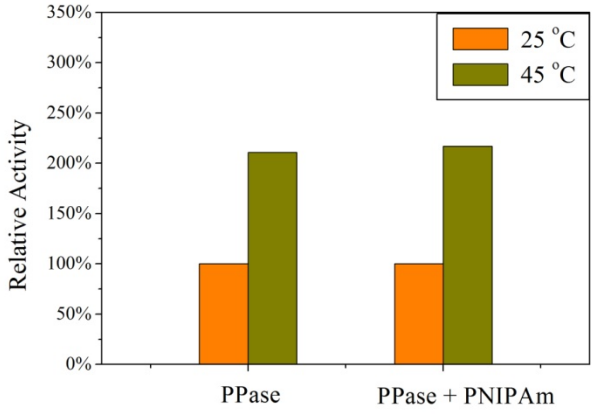

Fig. S9 Activity of PPase and PPase added with equal molar PNIPAm.

3.11.c Polymerization of NIPAm in the presence and absence of PPase without RAFT agent.

It should be declared that polymerization cannot be carried out in water with only NIPAm and visible light but no photo initiator. Control polymerizations were carried out as described previously without CTA as the replacement of PPase-macroCTA with PPase or nothing. Both solutions turned to a condition like gel in minutes and unable to stir, indicating uncontrollable polymerization without CTA.

The gel like mixture for control polymerization of PPase was dissolved in large amount of DIW and characterized by SDS-PAGE. As shown in Fig. S10, no conjugate was detected, which means polymers produced in the solution were not connected to PPase. The same result was seen in the mixture of PPase with free PNIPAm obtained by maleimide-CTA.

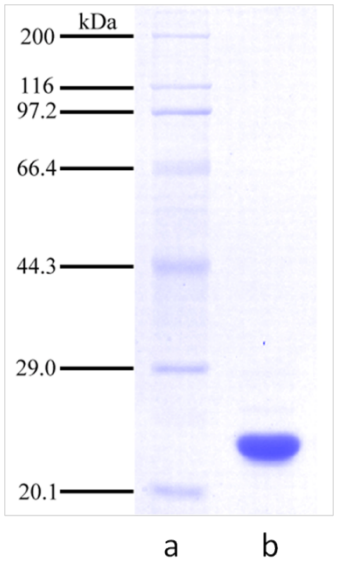

Fig. S10 SDS-PAGE result of control experiment. Lane a: molecular weight markers; lane b: control sample in the presence of PPase without the PPase-macroCTA.

3.12. Influence of CTA molecule attaching on PPase conformation.

Conformational changes of PPase conjugated with CTA were determined by CD to

determine the mechanism of protein activity change caused by CTA attaching. The CD signal from PPase in the 190-250 nm region was almost unchanged (Fig. S11a) but the CD signal in the 250-350 nm region was significantly altered (Fig. S11b) before and after CTA immobilization, suggesting that the CTA conjugation did not affect the secondary structure of PPase but showed a significant effect on the tertiary structure of PPase. This leads to a subtle change in the local conformation near the active site of the protein, which eventually has a considerable effect on the protein activity.

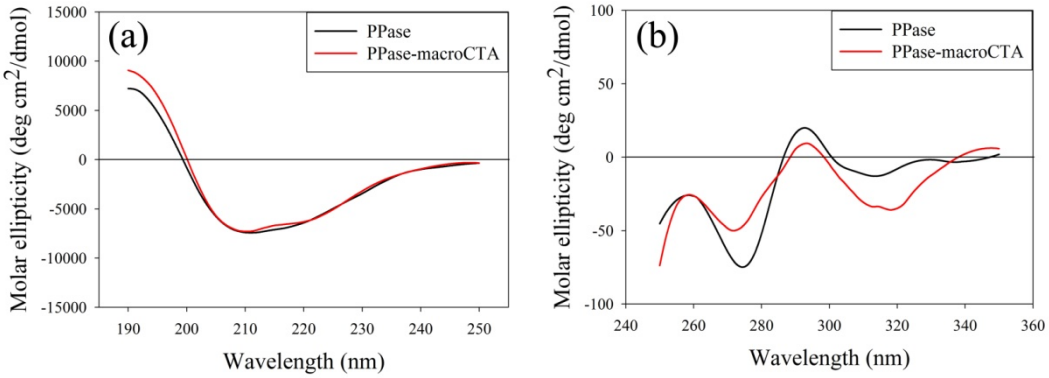

Fig. S11 CD spectra in the near ultraviolet region (a) and far ultraviolet region (b) of the PPase and PPase-macroCTA.

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