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Improvement in the Thermal Stability of Pyrophosphatase by Conjugation to Poly(*N*-isopropylacrylamide): Application to the Polymerase Chain Reaction

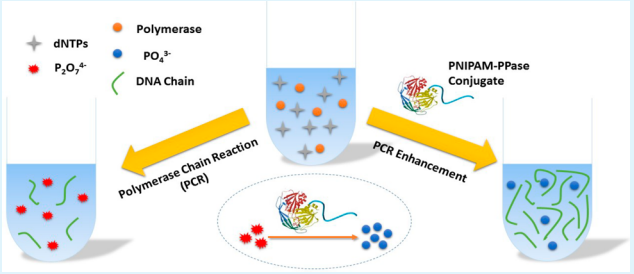
Yuecheng Cui,[†,§] Feng Liu,[†,§] Xin Li,[†] Lei Wang,[†] Hongwei Wang,[*,†] Gaojian Chen,[†] Lin Yuan,[†] John L. Brash,[‡] and Hong Chen[*,†]

[†]The Key Lab of Health Chemistry and Molecular Diagnosis of Suzhou, Department of Polymer Science and Engineering, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou 215123, People's Republic of China

[‡]School of Biomedical Engineering, Department of Chemical Engineering, McMaster University, Hamilton, Ontario L8S 4L8, Canada

Supporting Information

ABSTRACT: Polymerase chain reaction (PCR) is a powerful method for nucleic acid amplification. However, the PCR is inhibited in its yield due to its byproduct, pyrophosphate (PPi), a byproduct of the reaction; the yield is thereby limited. The conventional method for hydrolysis of PPi by pyrophosphatase (PPase) is not well adapted for operation at elevated temperatures over long times as required during the PCR. In this work, we reported a strategy to improve the PCR yield using a conjugate of the enzyme with the thermally responsive polymer poly(*N*-isopropylacrylamide) (PNIPAM). Pyrophosphatase (PPase) was conjugated to PNIPAM site-specifically near the active center. As compared to the free enzyme, the optimum temperature of the conjugate was shown to increase from 45 to 60 °C. For the conjugate, about 77% enzyme activity was retained after incubation at 60 °C for 3 h, representing a 6.8-fold increase as compared to the unconjugated enzyme. For the PCR using the conjugate, the yield was 1.5-fold greater than using the unconjugated enzyme. As well as improving the yield of the PCR (and possibly other biological reactions) at elevated temperature, polymer conjugation may also provide a strategy to improve the heat resistance of proteins more generally.



KEYWORDS: *poly(N-isopropylacrylamide), pyrophosphatase, protein activity, thermal stability, PCR enhancement*

1. INTRODUCTION

The polymerase chain reaction is a landmark technique that can simulate the DNA replication process in vitro. It is widely used in molecular biology, genetic engineering, and clinical medicine.[1] However, researchers have found that the PCR yield is limited by pyrophosphate ($P_2O_7^{4-}$, PPi) formed as a byproduct during the reaction and limiting its progress in the positive direction.[2] Hence, eliminating or reducing PPi is a valid approach for improving the yield of PCR.

Commonly, polyphosphate molecules, including PPi, hydrolyze quite slowly. Using chemical methods, hydrolysis of PPi is carried out at high concentrations of acid;[3] this is clearly inappropriate for the alkal conditions of the PCR.[1] However, pyrophosphatase (PPase), an enzyme found in most organisms, can hydrolyze PPi to phosphate (PO_4^{3-}, Pi) rapidly and effectively under mild conditions.[4] Therefore, the catalytic action of PPase has the potential to improve the PCR yield. A challenge in the use of PPase in the PCR is the requirement to maintain its hydrolytic activity at temperatures around 60 °C for 1−3 h. *Escherichia coli* (*E. coli*) bacteria can produce PPase with high catalytic activity of about 800 units/mg. However, the optimum temperature for *E. coli* PPase is about 45 °C,[5] above which PPase will be denatured after heat treatment for long time. Other candidates are the PPases with heat-resisting properties produced by thermophilic bacteria. A difficulty is to screen these microorganisms and then to prepare large amounts of PPase with high purity and specific activity. Recently, some commercial heat-resisting PPases have become available, for example, PPase from *Bacillus stearothermophilus*. However, their specific activity is much lower than that of PPase from *E. coli* and is not sufficient for most applications.[6−9] An alternative strategy is to modify *E. coli* PPase to increase its optimum temperature and improve its thermal stability.

A number of techniques have been used for protein modification, including conjugation to synthetic polymers. Conjugation protects the protein structure and regulates its catalytic activity depending on the properties of the polymer.[10,11] Poly(*N*-isopropylacrylamide) (PNIPAM) is widely used as a thermally responsive polymer, and has been applied for protein modification.[12] Chen et al.[13] prepared β-D-glucosidase conjugates using PNIPAM having an *N*-hydroxysuccinimide (NHS) ester functional group. The conjugated

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enzyme showed increased activity in the temperature range 45−65 °C, and its thermal stability was about twice that of the native enzyme at 60 °C. Cummings et al.[14] synthesized chymotrypsin−PNIPAM conjugates using the "grafting from" method; polymers were grown from a water-soluble initiator on the surface of the protein. The conjugates showed increased stability as compared to the native chymotrypsin. In the present work, it is hypothesized that conjugation of PPase to PNIPAM will improve the thermal stability of the enzyme as required to increase the PCR yield.

Because thermal denaturation of proteins is usually caused by the destruction of the protein structure near the active center, it seems appropriate in the conjugation reaction to attach the PNIPAM near the active center.[11,15,16] It is expected that this approach should inhibit loss of the protein native structure but have little influence on the protein surface properties.[17] Thus, PNIPAM was first synthesized by reversible addition−fragmentation chain transfer (RAFT) polymerization and then covalently attached specifically near the active center of *E. coli* PPase. The activities of PNIPAM−PPase conjugates were investigated at different temperatures and after thermal treatment for 10−180 min. Finally, the effect of PNIPAM−PPase conjugate on the PCR yield was studied.

2. MATERIALS AND METHODS

N-Isopropylacrylamide (NIPAM, Acros, 99%) was purified via recrystallization from a mixture of toluene/hexane (50%, v/v) and dried under vacuum before use. 2,2′-Azobis(isobutyronitrile) (AIBN, Aldrich, 98%) was recrystallized from ethanol and dried at room temperature in a vacuum oven. 4-(4-Cyanopentanoicacid) dithiobenzoate (CPDB, Aldrich, 98%), ethanolamine (TCI, 99%), 2,2′-dithiodipyridine (DTP, TCI, 98%), and dithiothreitol (DTT, Aldrich, 99.5%) were used as received. Dialysis membranes (molecular weight cutoff (MWCO): 3.5 kDa) were purchased from Solarbio Science & Technology Co., Ltd. The centrifuge filters (Amicon Ultra, MWCO 30 kDa) were obtained from Millipore Corp. Deionized water was used in all experiments, which was purified by a Millipore water purification system to give a minimum resistivity of 18.2 MΩ cm. λ DNA, DNA polymerase (PrimeStar HS), and restriction endonucleases, including *Pst*I, *Eco*RI, *Bam*HI, and *Hin*dIII, were purchased from Takara Biotechnology Co. All other chemicals were purchased from Shanghai Chemical Reagent Co. and used without further purification, unless otherwise specified.

Measurements. ^{1}H NMR spectra of the PNIPAM and functionalized pyridyl-PNIPAM were recorded on an INOVA 400 MHz nuclear magnetic resonance instrument, using D_2O as solvent and tetramethylsilane (TMS) as the internal standard. The number-average molecular weight (M_n) and molecular weight distribution (M_w/M_n, PDI) of the polymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument using a PL gel bead size guard column (50 × 7.5 mm^2 column, pore size 500 Å, particle size 5.0 μm, and Mixed-C) and a differential refractive index detector. Using DMF as the eluent, a series of poly(methyl methacrylate) standards with a flow rate of 1.0 mL/min at 30 °C was applied for calibration.

Generation of PPase Cys Mutant. Generation of PPase Cys mutant referred to previous literature.[18] Tersely, site-directed mutagenesis was carried out for cloning mutant *ppa* gene on the basis of the megaprimer PCR method.[19] The forward flanking primer sequence for the megaprimer PCR was 5′-CGCAAGCTTTTATTTATTCTTTGCGCGCTC-3′, and the reverse flanking primer sequence was 5′-CGCGGATCCAGCTTACTCAACGTCCCT-3′. The mutagenic primer for creating the K148C mutant was 5′-CCTCGAAAAAGGCTGCTGGGTGAAAGTTGAAGG-3′ (the underlined nucleotides are the mutated codons). The megaprimer PCR products were digested with *Bam*HI and *Hin*dIII and then ligated to the *Bam*HI−*Hin*dIII site of the pQE30 vector. The recombinant plasmids were transfected into *E. coli* XL1-Blue to express protein. *E. coli* XL1-Blue cells that expressed the PPase mutants were cultured in liquid LB medium for further IPTG induction. The resultant cell precipitates were disrupted with lysozyme and sonication to get PPase supernatant for further purification via Ni-NTA Sepharose resin (Shanghai Sangon Biotech Co., Ltd., China). The purified mutants were then concentrated with centrifuge filters (Amicon Ultra, MWCO 50 kDa) and verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 4% stacking gel and a 12% separating gel in a Mini-Protean II apparatus (Bio-Rad).

RAFT Polymerization of PNIPAM. PNIPAM was polymerized via RAFT polymerization as described in the literature,[20] using CPDB as the RAFT agent and AIBN as the initiator. Typically, 1.13 g of NIPAM was dissolved in 5 mL of *N*,*N*′-dimethylformamide in a glass round-bottom flask, with 28 mg of CPDB and 3.3 mg of AIBN. The mixture was purified by nitrogen for one-half an hour under stirring and then transferred into a glovebox. The polymerization was carried out at 65 °C for 24 h. The solution was observed to stay pink. The polymer was purified by dialysis tubing (MWCO = 3.5 kDa) and dialyzed for 4 × 3 h against water. The polymer was then lyophilized overnight and analyzed by ^{1}H NMR in D_2O and by GPC in DMF.

Pyridyl Disulfide-Functionalization of PNIPAM. The post-modification method referred to Davis's work.[21] In a typical procedure, 0.1 g of PNIPAM as polymerized before (M_n = 10 000 g/mol by GPC, PDI = 1.17) and 32 mg of DTP were dissolved in 2 mL of acetonitrile. The solution was purged with nitrogen for 30 min, and 1 mL of a solution of ethanolamine (18 mg in 1 mL of acetonitrile, 0.29 M) was added dropwise in a glovebox under nitrogen purge. The solution was observed to transform from pink to yellow and stirred for 4 h at room temperature. The polymer was dialyzed (MWCO = 3.5 kDa) against an acetone/water mixture (50/50 V%) for 2 × 3 h to remove excess 2,2′-dithiodipyridine, and dialyzed against water for 2 × 3 h to remove acetone. The polymer was lyophilized overnight and analyzed by ^{1}H NMR in D_2O. A similar procedure was used for all polymers.

Preparation of PNIPAM−PPase Conjugates. Typically, 143 mg of pyridyl disulfide-functionalized PNIPAM (14.3 μmol, M_n by GPC = 10 000 g/mol, PDI = 1.17) was dissolved in 5 mL of 50 mM Tris-HCl buffer, pH 8.0, and added dropwise to a PPase solution (0.286 μmol in 1 mL of H_2O). The mixture was incubated overnight at room temperature. The unreacted polymer was removed with Ni-NTA Sepharose resin (Shanghai Sangon Biotech Co., Ltd., China), and then centrifuged at 4 °C in 30 kDa molecular weight cutoff centrifuge filters (Amicon Millipore) at 7500*g* for 10 min six times. The purity of the PNIPAM−PPase conjugates was verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a 4% stacking gel and a 12% separating gel in a Mini-Protean II apparatus (Bio-Rad). Samples were prepared at 1 mg/mL in Tris buffer (pH 6.8) containing bromophenol blue and glycerol. The gels were dyed with Coomassie Brilliant Blue and analyzed with an EC3 imaging system.

Low Critical Soluble Temperature (LCST) Measurements. PNIPAM and PNIPAM−PPase conjugates were dissolved in deionized water at a concentration of 1 mg/mL overnight to ensure complete dissolution, respectively. The solutions were heated ranging from 25 to 35 °C with the rate of 5 min/°C and determined at each temperature degree by a UV−vis spectrophotometer at 490 nm. The LCST was determined to be the temperature at 10% of the maximum absorbance.

Activity Assay of PPase and PNIPAM−PPase. The activity of the PPase and conjugates was assayed as previously described in the literature.[22] For instance, using sodium pyrophosphate enzymatic as the substrate, the hydrolysis of PPase was performed at 30 °C for 10 min in 50 mM Tris-HCl buffer (pH 8.0) mixed with 0.2−3.5 μg/mL PPase, 50 mM $MgCl_2$, and 2 mM $Na_4P_2O_7$. The reaction (V = 0.1 mL) was terminated by adding 10 μL of 0.4 M citric acid, and then 0.8 mL of AAM solution (acetone-acidmolybdate) mixed with 10 mM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 2.5 M H_2SO_4, and acetone (25/25/50 v%) was added to the tubes. The color reaction was taken for 2 min, and 80 μL of 1 M citric acid was then added. The yellow color was determined with a spectrophotometer at 355 nm. The protein concentration was measured via the Bradford method. The protein

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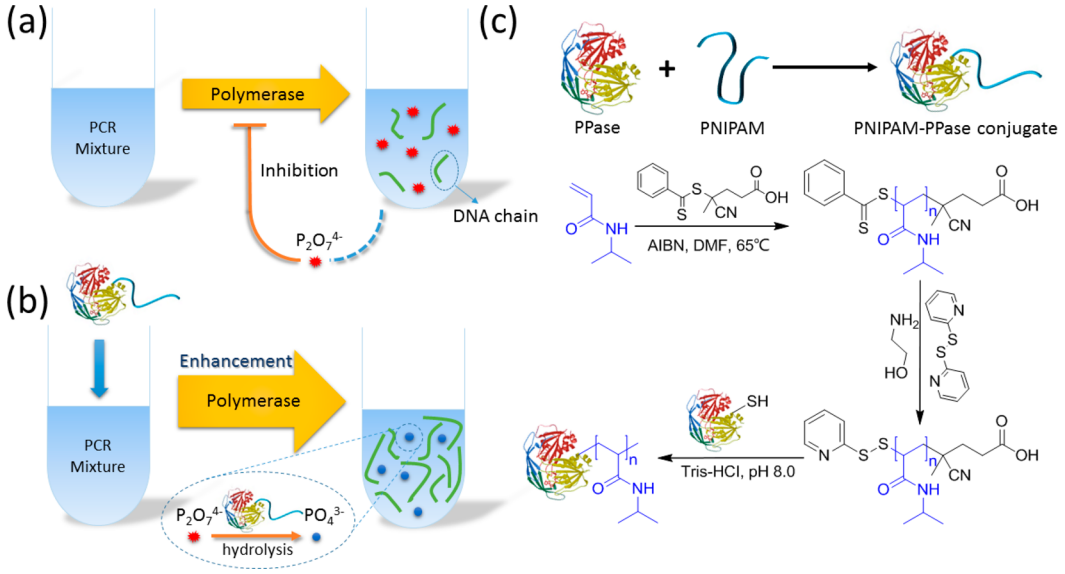
Scheme 1. (a) Normal PCR Procedure, Subject to Inhibition by the Product Pyrophosphate (PPi), (b) PCR Enhancement by Hydrolysis of PPi to Pi Using Heat-Resisting Pyrophosphatase, and (c) PNIPAM-PPase Conjugate Synthesis Strategy



activity is expressed in katal (kat = 1 mol/s) per kg protein. The activity of native PPase (control) was taken to be 100%.

From this value, the relative activity of PNIPAM−PPase conjugates was calculated.

Determination of the Temperature Curve and Thermal Stability. For the temperature curve, the PPase or PNIPAM−PPase conjugates (10 μL, protein concentration: 5 μg/mL) dissolved in 81 μL of 50 mM Tris-HCl buffer (pH 8.0) solution were mixed with 5 μL of 1 M $MgCl_2$. The solution was incubated with 4 μL of 50 mM $Na_4P_2O_7$ for 10 min at varying temperature from 25 to 90 °C for activity assay.

For the thermal stability, the protein or the conjugates (5 μg/mL of protein concentration) were first incubated in 50 mM Tris-HCl buffer (pH 8.0) at 60 °C for varying time from 10 to 180 min, and aliquots (10 μL) were removed after a certain time of incubation and diluted for the activity assay.

Polymerase Chain Reaction (PCR). The normal PCR reaction solution (V_{Total} = 50 μL) was composed of 5 μL of 10× PCR buffer, 2.5 mM dNTP mixture, 2.5 μL of (+)primer, 2.5 μL of (−)primer, 1 μL of template DNA, 4 μL of 25 mM $MgCl_2$, 0.5 μL of Taq DNA polymerase, and 29.5 μL of nuclease free water. The reaction solution of PNIPAM-PPase assisted PCR was composed of 5 μL of 10× PCR buffer, 2.5 mM dNTP mixture, 2.5 μL of (+)primer, 2.5 μL of (−)primer, 1 μL of template DNA, 4 μL of 25 mM $MgCl_2$, 0.5 μL of Taq DNA polymerase, 11.9 μL of 5 μg/mL PPase-PNIPAM, and 17.6 μL of nuclease free water. The primers used for the reaction were 5′-CGCGGATCCGAAAACTTTAAACATCTCCCT-3′ and 5′-CGCAAGCTTTTAAACTTCTTTAAGTTTTGCGGTG-3′. The PCR was carried out in a thermal cycler (Mastercycler ep gradient S, Eppendorf, Germany): 94 °C for 20 s; 20 cycles of 94 °C for 30 s, 60 °C for 90 s; and finally 60 °C for 10 min. After PCR amplification, the products were loaded on agarose gels (0.8%) for DNA electrophoresis (80 v, 75 min). The gels were stained with ethidium bromide and detected with an EC3 imaging system (UVP Inc., U.S.). The quantity of PCR product was calculated from the fluorescence intensity of DNA using ImageJ.

Ellman's Assay. The free thiol content was measured by Ellman's Assay.[23] The Ellman's reagent solution was prepared using 4.0 mg of Ellman's agent dissolved in 1 mL of 0.1 M sodium phosphate buffer (pH 8.0) including 0.1 mM EDTA. A 250 μL aliquot of protein sample with 5.0 mg/mL of concentration at least was mixed with 50 μL of Ellman's reagent solution and 2.5 mL of 0.1 M sodium phosphate buffer (pH 8.0) to react at room temperature for 20 min. The absorbance at 412 nm was measured with a spectrophotometer. The concentration of thiol group was calculated via Beer−Lambert's law, with a molar extinction coefficient for 2-nitro-5-thiobenzoic acid of 14 150 M^{-1} cm^{-1} at 412 nm.

3. RESULTS AND DISCUSSION

Synthesis of PNIPAM−PPase Conjugates. The thiol side chain of cysteine residues is often used in site-specific polymer modification of proteins due to its high reactivity and relatively rare occurrence. Because there is no free cysteine residue on the surface of *E. coli* PPase, site-directed mutagenesis was used to generate a mutant (K148C) with a cysteine residue in position 148 to replace the original lysine residue near the active center.[20,22,24,25] Subsequently, the thiol group of cys^{148} was used to conjugate to PNIPAM by "grafting to" (Scheme 1).[17,26] The enzyme was conjugated to PNIPAM in the final step via simultaneous aminolysis and thiol−disulfide exchange reactions, so that maximum activity could be retained.[21,27]

It has been reported that the molecular weight of the conjugating polymer chain may influence the properties of the conjugate.[28] Therefore, PNIPAM−PPase conjugates were synthesized using three PNIPAM molecular weights (7300, 21 000, and 66 000 Da); molecular weight variation was effected by regulating the feed ratio NIPAM:CTA[29] (Table 1). The polymers were characterized by ^{1}H NMR and GPC

Table 1. Properties of PNIPAM

sample	$[M]_0/[CTA]_0/[I]_0$	$M_{n,GPC}$	PDI
P_1	110.0/1/0.2	7300	1.13
P_2	220.0/1/0.2	21 000	1.17
P_3	650.0/1/0.2	66 000	1.15

(Figures S1 and S2). The conjugates were formed by thiol−disulfide exchange reaction and characterized using sodium dodecyl sulfonate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figures S3−S5). After conjugation, the lower critical soluble temperature (LCST) of conjugates was a little higher than that of pure PNIPAM due to the hydrophilicity of PPase.[18]

Temperature-Dependent Activity of PNIPAM−PPase Conjugates. The catalytic activity of PPase (hydrolysis of PPi

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to Pi) after conjugation to PNIPAM did not change. Also, in the temperature range from 25 to 45 °C, there was no significant difference in the specific activity of PPase and PNIPAM-PPase. However, at temperatures above 50 °C, the conjugates showed higher specific activity. This may be related to the change of the optimum temperature of PPase after conjugation to PNIPAM. Similarly to previous reports,[5,9] the optimum temperature of PPase was found to be about 45 °C, the activity of which is 11.13 ± 0.13 kat/kg, decreasing rapidly to 8.39 ± 0.08 kat/kg at 60 °C. Interestingly, the PNIPAM-conjugated PPase showed different behavior: as the temperature increased to 50−60 °C, the specific activity of the conjugates did not decrease, but rather increased to 12.08 ± 0.31 kat/kg, indicating an optimum temperature of about 60 °C (Figure 1).

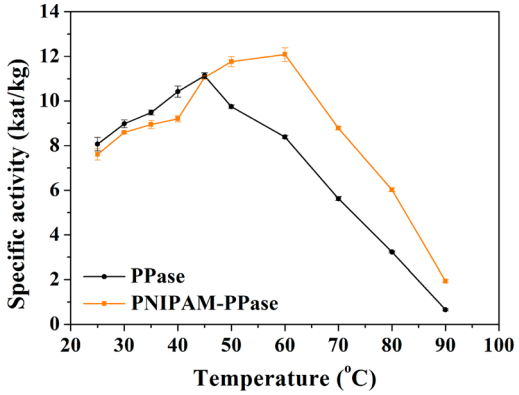

Figure 1. Specific activity versus temperature of free PPase and PNIPAM−PPase conjugate as a function of temperature. Mean ± SD, n = 3.

Comparing the relative specific activities of PPase and PNIPAM−PPase (taking the specific activity of unconjugated enzyme as 100%), we showed that the activity of both enzymes was similar in the range of 20−45 °C. With further increase in temperature, the relative activity of PNIPAM−PPase increased. At a temperature of 90 °C, the relative activity of the conjugated enzyme was almost 3-fold greater than that of the free enzyme (Figure 2). These data indicate that the catalytic efficiency of the enzyme at higher temperature can be increased by PNIPAM conjugation.

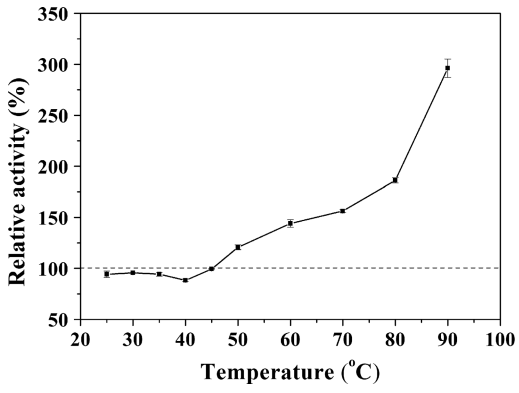

Figure 2. Activity versus temperature of PNIPAM−PPase conjugate relative free PPase as a function of temperature. Mean ± SD, n = 3.

Thermal Stability of the Conjugates. In addition to the increase in optimum temperature, the thermal stability of PPase after conjugation to PNIPAM was considerably improved. Thus, after incubation at 60 °C for 60 min, the specific activity of unconjugated PPase decreased to 4.17 ± 0.03 kat/kg, constituting a loss of about 50% (Figures 3 and 4); activity

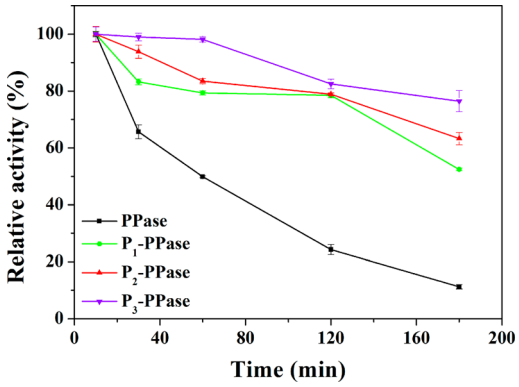

Figure 3. Relative activities of free PPase and PNIPAM−PPase conjugates of different PNIPAM molecular weight (Mn(PNIPAM) = 7, 21, and 66 kDa for P_1, P_2, and P_3, respectively) as a function of incubation time. Mean ± SD, n = 3.

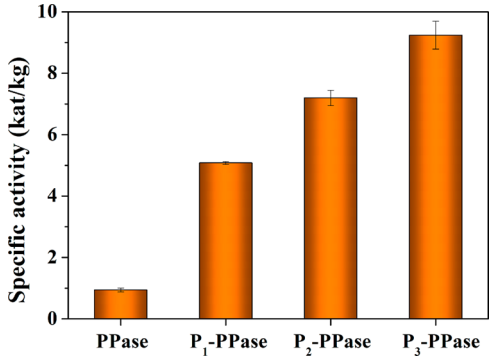

Figure 4. Specific activity of PPase and P_1, P_2, P_3−PPase conjugates after incubation for 3 h at 60 °C. Mean ± SD, n = 3.

continued to decrease with time at the higher temperature. After 180 min, the activity decreased by about 80% to 0.94 ± 0.06 kat/kg. These data show that the unconjugated PPase is quite unstable at higher temperature.

However, after PNIPAM conjugation the enzyme stability increased dramatically. The activities of the PNIPAM−PPase conjugates were 4.7−6.8 times greater than that of the unconjugated enzyme after incubation at 60 °C for 180 min. Furthermore, the increase in stability was greater for higher molecular weights of PNIPAM. For PNIPAM molecular weights of 7300 Da (P_1) and 21 000 Da (P_2), the activity decreased to 83.5 ± 1.0% and 79.4 ± 0.7%, respectively, after 60 min at 60 °C, and to 52.4 ± 0.5% and 63.3 ± 1.9%, respectively, after 180 min. When the molecular weight of the PNIPAM was increased to 66 000 Da (P_3), the specific activity remained high at 11.86 ± 0.11 kat/kg after 60 min. There was no significant difference in activity after 60 min as compared to an unheated control sample, and both showed activities higher than that of the free enzyme. Furthermore, after incubation for 180 min, the activity of the conjugated enzyme was 9.24 ± 0.45 kat/kg; that is, it retained almost 80% of its activity. However, the activity of PPase conjugated PNIPAM with larger molecular

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weight of 78 000 Da showed a slight decrease after 10 min incubation at 60 °C (Figure S6). These data indicate that when conjugated to PNIPAM, PPase retains its catalytic activity over time at high temperature, possibly due to the facilitation of protein and substrate binding provided by the hydrophobic polymer chains. On the other hand, the conformation of the PNIPAM at higher temperature may be such that the enzyme structure near the active center is protected. The collapsed state would dampen the structural dynamics of the protein.[14,29]

PCR Enhancement Using PNIPAM−PPase Conjugates. Because the P_3−PPase conjugate exhibited the highest thermal stability, this material was chosen to investigate the effect of conjugation on the PCR yield. As seen in agarose gels (Figure 5a), the yield of PNIPAM−PPase assisted PCR was greater

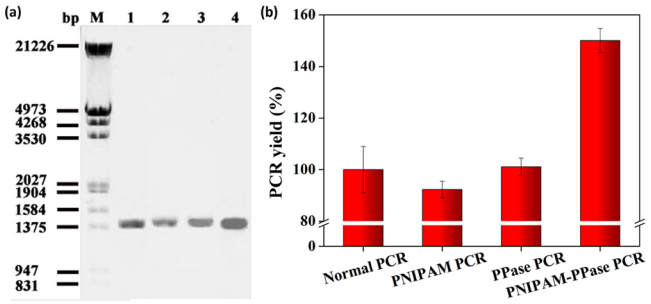

Figure 5. PCR enhancement using PNIPAM−PPase conjugates: (a) agarose gel electrophoresis: lane 1, normal PCR; lane 2, PNIPAM PCR; lane 3, PPase PCR; lane 4, PNIPAM-PPase PCR; (b) yield of normal PCR, PNIPAM PCR, PPase PCR, PNIPAM-PPase PCR. Mean ± SD, n = 3.

than that of the conventional PCR, the increase being of the order of 50%. Furthermore, there were no nonspecific amplifications formed in the PNIPAM−PPase PCR, showing that the PCR did not lose specificity when using PNIPAM−PPase conjugate (Figure 5a). However, after adding PPase, the gel of the product showed no significant change, and the yield was 101.1 ± 3.4% (Figure 5a,b and Figure S7). It also can be seen that the yield of the PNIPAM assisted PCR decreased slightly to 92.3 ± 3.2%, suggesting that PNIPAM may inhibit the PCR (Figure 5a,b and Figure S7). These results indicate that the PNIPAM−PPase conjugates can stimulate the PCR without changing the specificity, presumably due to its high activity at higher temperature and a similar optimum pH value (about pH 8.0).

Thermal Stability of the Conjugates in Different Cycles of PCR. The PCR usually has a lifetime of about 30 thermal cycles; however, some special types of PCR require a greater number.[30] To investigate the PNIPAM−PPase conjugates from this point of view, the activity of the conjugates after differing numbers of cycles was investigated. After 20 cycles, the specific activity of PNIPAM−PPase remained high at 9.68 ± 0.26 kat/kg. No significant difference in specific activity was observed after 80 cycles (P = 0.242), and after 100 cycles it decreased to 7.60 ± 0.08 kat/kg. These data suggest that the PNIPAM−PPase conjugates have the potential to be used in PCR applications requiring higher numbers of thermal cycles (Figure 6).

4. CONCLUSIONS

PNIPAM−PPase conjugates were prepared via site-specific modification near the active center of PPase. After conjugation

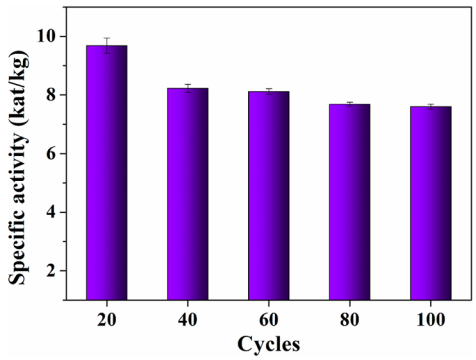

Figure 6. Specific activity of PNIPAM−PPase conjugate after repeated use in the PCR (expressed as number of cycles). Mean ± SD, n = 3.

with PNIPAM, the optimum temperature of PPase increased from 45 to 60 °C. In addition, the thermal stability of the conjugates was greatly improved. Stability increased with the molecular weight of the PNIPAM in the range of 7−66 kDa. The improved thermal stability of PNIPAM−PPase suggested that its use in the PCR would be advantageous. Thus, it was shown that in the presence of PNIPAM−PPase conjugate, the PCR yield increased by a factor of 1.5 as compared to conventional PCR after 20 cycles, and the specificity of the reaction was unchanged. The effect of PNIPAM−PPase on the PCR may be due to the maintenance of its specific activity over multiple PCR thermal cycles. It was shown that even after 100 cycles, about 80% of the initial PNIPAM−PPase activity remained, indicating the potential for applications requiring higher numbers of cycles. These advantages of the PNIPAM−PPase conjugate might be used to amplify and detect a very low amount of DNA. It is expected that the use of polymer−protein conjugates may not only improve the stability of the proteins (and other biomolecules) under harsh conditions, but also should be favorable for applications in biological reactions, enzyme engineering, and other related fields.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b06494.

^{1}H NMR and GPC of PNIPAM; LCST measurements of PNIPAM before and after conjugation; SDS-PAGE result of conjugation; the specific activity of PNIPAM−PPase conjugates of different polymer molecular weight; free thiol content of K148C, conjugates, and wild-type PPase and PCR yields of PNIPAM PCR, PPase PCR, and PNIPAM and PPase PCR (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: wanghw@suda.edu.cn.

*Tel.: +86-512-65880827. Fax: +86-512-65880583. E-mail: chenh@suda.edu.cn.

Author Contributions

§Y.C. and F.L. contributed equally.

Notes

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