Article

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Recyclable Thermoresponsive Polymer−β-Glucosidase Conjugate with Intact Hydrolysis Activity

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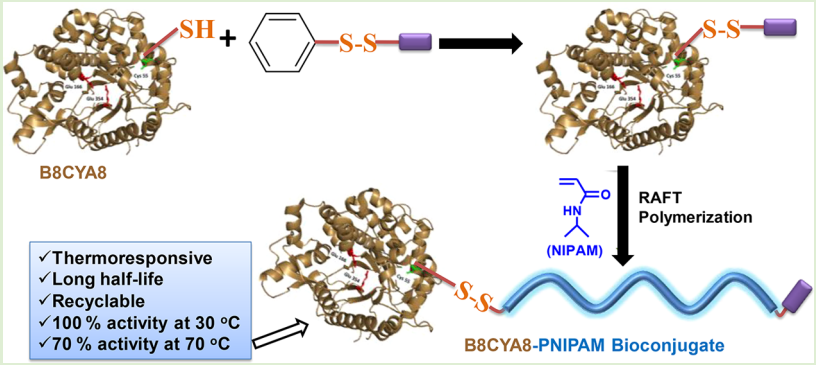
Supporting Information



ABSTRACT: β-Glucosidase (BG) catalyzes the hydrolysis of cellobiose to glucose and is a rate-limiting enzyme in the conversion of lignocellulosic biomass to sugars toward biofuels. Since the cost of enzyme is a major contributor to biofuel economics, we report the bioconjugation of a temperature-responsive polymer with the highly active thermophilic β-glucosidase (B8CYA8) from *Halothermothrix orenii* toward improving enzyme recyclability. The bioconjugate, with a lower critical solution temperature (LCST) of 33 °C withstands high temperatures up to 70 °C. Though the secondary structure of the enzyme in the conjugate is slightly distorted with a higher percentage of β-sheet like structure, the stability and specific activity of B8CYA8 in the conjugate remains unaltered up to 30 °C and retains more than 70% specific activity of the unmodified enzyme at 70 °C. The conjugate can be reused for β-glucosidic bond cleavage of cellobiose for at least four cycles without any significant loss in specific activity.

INTRODUCTION

Hydrolysis of cellulose in the highly heterogeneous lignocellulosic biomass requires the synergistic action of a minimum set of three cellulase enzymes: endoglucanase, cellobiohydrolase, and β-glucosidase. The fermentable sugars produced can be further fermented by microbes to produce biofuels. The efficient and economically feasible conversion of lignocellulose to biofuels still requires technical improvements before larger scale commercialization. One important limitation of this process is the high cost of the enzymes involved in the conversion of cellulose into fermentable sugars,[1,2] which significantly contributes to the economics of biofuel production. Methods to reuse the enzymes multiple times during subsequent hydrolysis cycles may lead to a reduction in enzyme production cost and in turn the economics of biofuel production. The recycling enzymes, while a desirable goal, are feasible only when the enzyme is stable through several cycles.[3,4] The long hydrolysis reactions require thermostable enzymes that are active during the time of the reaction.

Two strategies to recover cellulases include recovery of enzyme fraction present in the liquid phase and the other fraction bound to the solid lignocellulosic substrate. Free cellulases in the hydrolysis media have been reported to be recovered by its readsorption on fresh substrate.[5,6] The other strategy of recovering solid bound substrate include recycling the residual lignin that the enzyme adventitiously binds. However, the increase in lignin amounts during the recycling rounds makes this more difficult.[7] Other approaches include the use of surfactants, alkali, urea, glycerol, polyethylene glycol, and so on.[8,9] Another well-known and comparatively uncomplicated traditional technique is the bioconjugation of synthetic polymers to biomolecules such as proteins and peptides to immobilize enzymes.[10,11] Attachment of proteins to polymers generally leads to improved pharmacological behavior such as longer half-life, reduced proteolytic degradation, slower clearance rate, and so on in therapeutic applications.[12,13] Covalent attachments of biocompatible poly(ethylene glycol)

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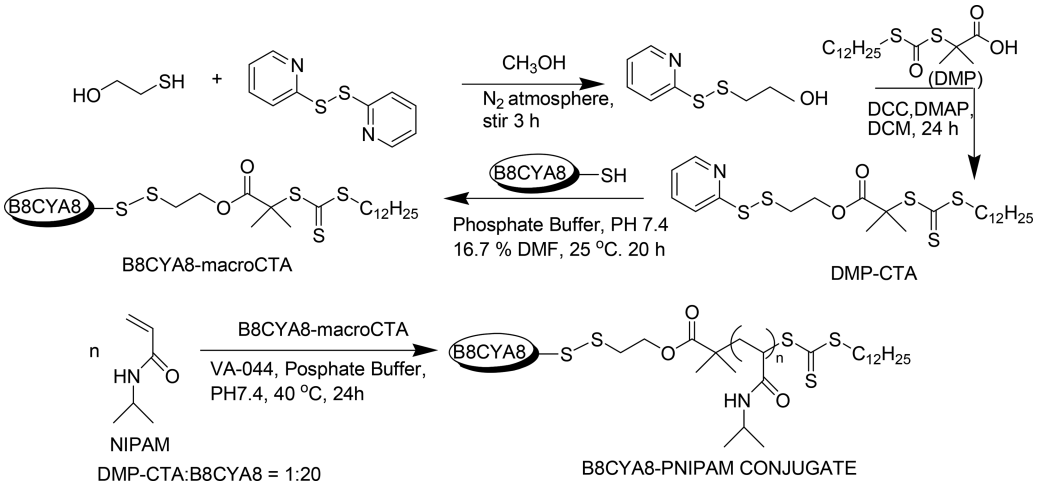
Scheme 1. Synthesis of Pyridyl Disulfide Terminated DMP-CTA, Modification of B8CYA8 with the CTA Moiety, and *grafting from* RAFT Polymerization of NIPAM in the Presence of B8CYA8-macroCTA



(PEG) chains to proteins, termed as "pegylation", are already well explored in this field.[14,15] Liu et al. recently reported a fluorogenic coupling of protein/antibody bioconjugates through incorporation of reactive azide functionality to the PEG terminus via click reaction.[16] Pegylation however has several drawbacks, like a decrease in protein activity after PEG attachment, polymeric accumulation in liver and spleen after long-lasting treatment of pegylated therapeutics, hypersensitivity etc.[17,18] Hence, new and improved approaches of protein-zwitterionic polymer bioconjugates as a promising PEG alternative have been reported.[19,20] Stimuli-responsive polymers as a better replacement of pegylation method are being extensively studied in the literature.[21,22] The temperature-responsive polymers exhibiting lower critical solution temperature (LCST) behavior that can be adjusted by changing the hydrophilicity of the material and have important applications in the progress of "smart" materials for therapeutic use.[23,24] In particular, poly(*N*-isopropylacrylamide) (PNIPAM),[25,26] poly-[oligo(ethylene glycol) (meth)acrylate]s,[27] and poly(2-oxazoline)s[28] have been rigorously investigated for biological applications due to their biocompatibility and the ability to use LCST by introducing more hydrophilic or hydrophobic comonomers. Furthermore, the resulting temperature-responsive polymer–protein conjugates can be easily purified by thermal precipitation and are another advantage of using such LCST exhibiting polymers.

Several approaches, like *grafting from*, *grafting to*, and *grafting through*, are being explored for the preparation of polymer–protein conjugates.[10,29] More facile conjugation strategy is derived from protein modification and proliferation of polymer chain from protein surface (*grafting from* method). The advantage of this approach lies in the simplicity of purification after easy removal of unreacted monomers and other impurities.[30] Amine, carboxylic acid, hydroxyl, and thiol groups are generally observed as reactive functionalities in the biomolecules.[31] Thus, various types of bioconjugates have been researched with many different proteins/enzymes and polymers.[32] Although there are several reports of polymer–protein or polymer–peptide conjugates, only a few polymer–enzyme conjugates have been reported.[33,34] Lysozyme is the most commonly used enzyme with the lysozyme–polymer conjugates prepared via amide linkages through free $-NH_2$ group present in the enzyme active side.[35,36] Only a few β-glucosidase–polymer conjugate have been reported in the literature.[37]

Datta and co-workers recently reported the recombinant cloning and characterization of a cellulase component, the thermophilic GH1 β-glucosidase (B8CYA8) from *Halothermothrix orenii* with high specific activity and thermostability.[38] Previously, Mackenzie et al. reported a thermoresponsive hyperthermophilic endoglucanase bioconjugate through transamination of the enzyme where the authors demonstrated the generation of similar glucose equivalents as the unmodified enzyme.[39] Here we report the modification of a highly active B8CYA8 enzyme with a chain transfer agent (CTA) moiety through Cys residue for reversible addition–fragmentation chain transfer (RAFT) polymerization of *N*-isopropylacrylamide (NIPAM) monomer to prepare a B8CYA8–poly(*N*-isopropylacrylamide) (B8CYA8-PNIPAM) conjugate with high enzyme activity, thermal stability, long half-life, and reusability across multiple rounds of saccharification reactions after separation from the reaction mixture, without much change in specific activity upon recycling across four cycles of hydrolysis. Incorporation of such stimuli-responsive or "smart" polymers could be a useful strategy for solubility modification to enable enzyme recycling, in response to external stimuli, such as temperature.

MATERIALS AND METHODS

Materials. Hydroxypropyl-mercaptopyridine[40] and 2-dodecylsulfanylthiocarbonylsulfanyl-2-methyl-propionic acid (DMP)[41] were prepared following previously reported literature. 2,2′-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) was purchased from Wako Pure Chemical Industries, Osaka, Japan, and was recrystallized twice from methanol. *N*-Isopropylacrylamide (NIPAM, Sigma-Aldrich) was recrystallized using hexane. 4-Dimethyl aminopyridine (DMAP, 99%), anhydrous *N*,*N*-dimethylformamide (DMF, 99.9%), dicyclohexyl carbodiimide (DCC, 99%), and 2-hydroxyethyl methacrylate (HEMA, 97%) were obtained from Sigma-Aldrich, Bangalore, India. $CDCl_3$ (99.8% D) and D_2O (99% D) were purchased from Cambridge Isotope Laboratories, Inc., Tewksbury, U.S.A., for NMR study. Spectra/Por^R dialysis membrane with molecular weight cutoff (MWCO) of 2 kDa was used for dialysis. The pBAD bacterial expression plasmid was obtained from Thermo Fisher Scientific, Waltham, U.S.A. *Escherichia coli* Top 10F′ cells were purchased from Life Technologies, La Jolla, CA, U.S.A. A total of 30 kDa cutoff size Amicon-Ultra-15 membranes were obtained from EMD Millipore, Billerica, U.S.A. Bradford reagent and bovine serum

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albumin (BSA) for protein purification were obtained from Sigma-Aldrich, St. Louis, U.S.A., and Sisco Research Laboratories, Bangalore, India, respectively. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) marker was purchased from Thermofisher Scientific, Mumbai, India.

Instrumentation. ^{1}H NMR spectroscopy was performed on a 500 MHz Bruker Avance 500 spectrometer. Molecular weights and molecular weight distributions (dispersity ($Đ$)) of polymer protein conjugates were measured on a ACQUITY Advanced Polymer Chromatography (APC), Waters Corp, Milford, U.S.A., with an ACQUITY refractive index (RI) detector using a ACQUITY APC XT 2002.5 μm (4.6 × 7.5 mm) column at 45 °C. Samples were eluted at a flow-rate of 0.25 mL/min with water. Dextrin standards were used to calibrate the instrument. Matrix-assisted laser desorption/ionization−time-of-flight (MALDI-TOF) mass spectrometry was performed on a Bruker Ultrafle Xtreme instrument equipped with a smart beam-II laser in the reflector mode and an acceleration voltage of 22 kV, with sinapinic acid as the matrix. Sample solutions were prepared in phosphate buffer (pH 7.0). UV−vis spectroscopic study was performed on a PerkinElmer Lambda 35 spectrophotometer. Dynamic light scattering (DLS) study was performed on a Zetasizer Nano ZS (Malvern Instrument Ltd., U.K.) equipped with a He−Ne laser beam at 658 nm. Circular dichroism (CD) spectra were measured at 20 ± 1 °C with a JASCO J-815 CD spectrometer (Jasco, Tokyo, Japan).

Synthesis of Pyridyl Disulfide Terminated Chain Transfer Agent (CTA). Pyridyl disulfide-terminated DMP-CTA was synthesized (Scheme 1) as per the following protocol: DMP (2.0 g, 5.48 mmol) was dissolved in dry dichloromethane (DCM) and the solution was purged with dry N_2. The DCC (1.24 g, 6.03 mmol) and DMAP (0.13 g, 1.09 mmol) were subsequently added to the system. Then, hydroxypropyl-mercaptopyridine (1.02 g, 5.48 mmol) was added dropwise to the stirred reaction mixture cooled by ice−water bath. After 30 min, the reaction mixture was stirred at room temperature for 24 h. After filtering the insoluble *N,N′*-dicyclohexylurea, and an additional 80 mL of distilled water was added to the filtrate and then extracted with DCM. The organic layer was further washed with $NaHCO_3$ and brine solution and dried over Na_2SO_4. The solvent was removed by rotary evaporation and further purified by silica gel column chromatography using hexane/ethyl acetate as the mobile phase, yield = 76%.

Expression and Purification of B8CYA8. Gene encoding the β-glucosidase, B8CYA8, was cloned in pBAD bacterial expression plasmid and expressed in *Escherichia coli* Top 10F′ cells. Protein was purified as described earlier.[42] The purified protein was dialyzed against 10 mM potassium phosphate buffer, pH 7.0, and concentrated using 30 kDa cutoff size Amicon-Ultra-15 membranes.

Protein Quantification. Protein and protein−polymer conjugate concentration was measured by Bradford assay as per manufacturer's instructions.[43] The protein concentration in the conjugate was reported as mg protein/mL of conjugate.

Conjugation of B8CYA8 with Pyridyl Disulfide Terminated DMP-CTA to Obtain B8CYA8-MacroCTA. B8CYA8 (19 mg, 0.358 μmol, 1 equiv) was dissolved in 1 mL phosphate buffer solution (pH 7.4) in a 20 mL glass vial. The solution was purged with dry nitrogen for 40 min. A solution of pyridyl disulfide terminated DMP-CTA (3.82 mg, 7.17 μmol, 20 equiv) in nitrogen-purged DMF (200 μL, 16.7% DMF concentration in solution) was added to the enzyme, and the solution was stirred at room temperature for 20 h. The reaction mixture was centrifuged twice (at 4100 rcf for 20 min at 5 °C) to remove excess DMP-CTA. Subsequently, the supernatant was dialyzed against deionized (DI) water (6 × 18 mL) for 36 h using a membrane with MWCO of 2000 Da. The solution concentration was determined by UV−vis spectroscopy.

RAFT Polymerization of NIPAM Using B8CYA8-MacroCTA. NIPAM (21 mg, 0.186 mmol) and B8CYA8-macroCTA (10 mg, 0.186 μmol) in phosphate buffer, pH 7.4 (2 mL), were sealed in a 20 mL glass vial equipped with a magnetic stir bar and purged with dry nitrogen for 30 min in an ice bath. A concentrated and nitrogen-purged solution of VA-044 (0.06 mg in 0.2 mL) in phosphate buffer, pH 7.4, was added by syringe, and the reaction vial was placed in a reaction block preheated to 40 °C. VA-044 was employed at a ratio of [VA-044]/[CTA] = 1:1. The polymerization was quenched after 24 h.

RAFT Polymerization Kinetics. Two different polymerization conditions ([VA-044]/[B8CYA8-macroCTA] = 1:1 or 2:1) were studied to understand the effect of stoichiometry and B8CYA8 solution concentration on polymerization rate in phosphate buffer, pH 7.4 at 40 °C. Conversion of NIPAM was determined from the integration ratio of the vinyl protons at 6.02−6.15 ppm (2H from NIPAM monomers) to the isopropyl $-CH-(CH_3)_2$ area of both monomer and PNIPAM at 3.7−3.9 ppm.

Temperature Induced Precipitation of B8CYA8-PNIPAM Conjugate. After the RAFT polymerization of NIPAM with the B8CYA8-macroCTA, unconjugated B8CYA8 was removed from the B8CYA8-PNIPAM conjugates by thermally induced precipitation. A solution of conjugate in deionized (DI) water was heated at 40 °C for 10 min, followed by centrifugation at 13,600 rcf for 1 min. The supernatant was decanted, and the precipitate was dissolved in DI water at room temperature (~25 °C). The above procedure was repeated (4×) to remove unconjugated B8CYA8 from the B8CYA8-PNIPAM bioconjugates. Finally, the precipitate was dissolved in DI water.

Native Gel Electrophoresis. Native gel electrophoresis was done according to the standard Laemmli (1970) procedure in 10% polyacrylamide gels. The molecular weight marker used was PageRuler Plus Pre-Stained SDS PAGE marker. For protein visualization, gels were stained with Coomassie brilliant blue.

UV−Vis Spectroscopy. Native B8CYA8 showed absorbance at 280 nm. A total of 0.2 mg/mL of B8CYA8, B8CYA8-macroCTA, and purified B8CYA8-PNIPAM conjugate solutions were taken for absorbance measurement. The samples were placed to a quartz cuvette to measure the absorbance from 200 to 600 nm by a UV−vis spectrometer. The cuvette containing B8CYA8-PNIPAM conjugate was warmed from 24 to 40 °C (at different temperature intervals) over 30 min in a preheated temperature block equipped with a digital thermometer. The cuvette was quickly taken away from the preheated temperature block and the absorbance at 500 nm was measured by a UV−vis spectrometer. The phase transition temperature was defined as the temperature when 50% of the maximum transmittance was observed.

Dynamic Light Scattering (DLS). A freshly prepared B8CYA8-PNIPAM solution in DI water (0.2% w/v) was stored at 4 °C in refrigerator for 24 h. The solution was filtered through a 0.45 μm syringe filter at room temperature, and DLS measurements were carried out at 25 and 45 °C. The temperature of the solutions was regulated within an error of ±0.1 °C. A similar experiment was performed with native enzyme solution of same concentration.

Circular Dichroism. The solutions were made in 10 mM sodium phosphate buffer of pH 7.0 and spectra were recorded in a cuvette cell with path length of 0.1 cm (bandwidth = 1.0 nm; step resolution = 0.1 nm; scan speed = 100 nm/min; response time = 0.25 s) under constant nitrogen flow.

Enzyme Activity. Activity of enzyme-polymer complex was determined by using chromogenic substrate *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlc) containing the nonphysiological chromogenic aglycone *p*-nitrophenol (*p*NP) as per a protocol reported earlier.[38,44] For activity measurements, a 100 μL solution containing B8CYA8-PNIPAM conjugate (equivalent to 0.2 μg of protein) and 20 mM of *p*NPGlc in phosphate buffer, pH 7.0, was incubated at different temperatures between 20 to 70 °C for 5 min. The spontaneous hydrolysis of the substrate in the absence of B8CYA8-PNIPAM was subtracted as blank for all assays. All measurements were performed in triplicate.

Activity of Recycled B8CYA8-PNIPAM Conjugates. The activity of the recycled B8CYA8-PNIPAM was measured using *p*NPGlc as a substrate. For recycling assays, a 200 μL the reaction mixture contained 80 μg of B8CYA8-PNIPAM conjugate and 5 mM of *p*NPGlc (final concentration) and was incubated at 50 °C for 5 min with constant stirring at 500 rpm. To precipitate the B8CYA8-PNIPAM conjugate, the reaction contents were centrifuged at 10000 rpm for 1 min and the product was decanted. To solubilize the

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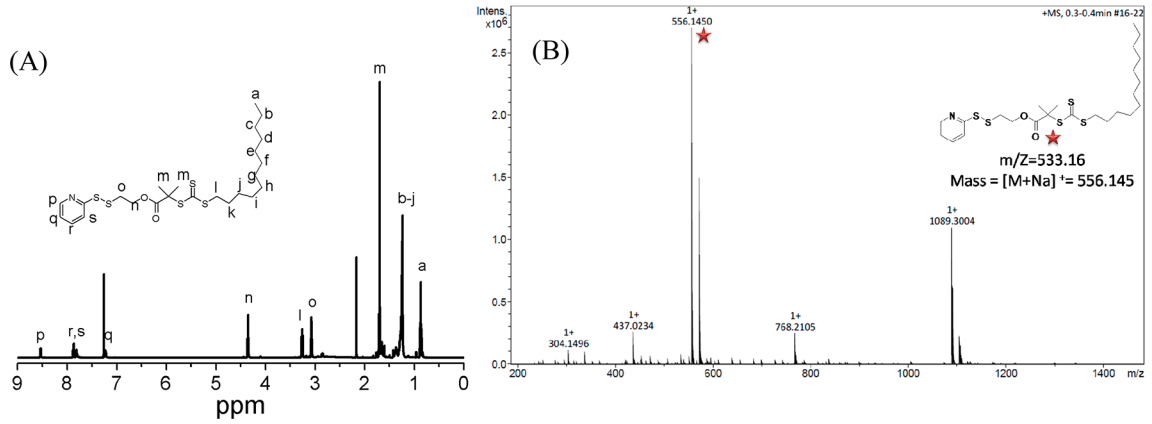

Figure 1. (A) ^{1}H NMR spectrum of DMP-CTA in $CDCl_3$ and (B) ESI-MS spectrum of DMP-CTA (calcd for [M + Na^+], 556.16 *m*/*z*; observed, 556.145 *m*/*z*).

precipitated conjugate, ice-cold phosphate buffer (pH 7.0) was added and assay procedures were repeated for activity measurement. A small fraction of solubilized conjugate was also taken out for quantification at the beginning of each cycle. This assay was repeated until the fourth cycle of precipitation. The concentration of the conjugate was determined in the solution before the start of each cycle of the activity assay, and the specific activity was calculated for each cycle based on the measured conjugate amount for each cycle.

Half-Life. A 100 μL reaction mixture of enzyme−polymer conjugate in 10 mM phosphate buffer, pH 7.0, was incubated at 70 °C and aliquots were taken out at regular time intervals, cooled, and assayed for residual specific activity. Half-life was calculated using the equation for linear decay.[45]

RESULTS AND DISCUSSION

Synthesis of B8CYA8-MacroCTA. Cys 55 in B8CYA8 is located away from the catalytic residues (Glu 166 and Glu 354) and displayed on the surface of the protein (Figure S1). This location would be expected to facilitate conjugate formation with minimum interference in catalytic activity. To utilize this one free sulfhydryl (−SH) group,[46] we chose to prepare B8CYA8-macroCTA from pyridine disulfide terminated CTA, followed by the reaction with the B8CYA8-SH, which easily removes 2-pyridinethione and binds B8CYA8 to CTA through a disulfide linkage.[12,47] We prepared the pyridine disulfide terminated CTA (DMP-CTA) by reacting DMP[41] with hydroxypropyl-mercaptopyridine[43] through DCC/DMAP coupling (Scheme 1). The formation of DMP-CTA was confirmed by ^{1}H NMR (Figure 1A) and electrospray ionization mass spectrometry (ESI-MS; Figure 1B). As the disulfide group in DMP-CTA showed high reactivity toward thiol functionality through the formation of disulfide linkage,[48] the reaction of B8CYA8 with DMP-CTA produced B8CYA8-macroCTA (Scheme 1). The enzyme modification by DMP-CTA was studied through UV−vis spectroscopy (Figure 2). Native B8CYA8 showed absorbance at 280 nm (Figure 2), but after modification with DMP-CTA, an additional peak at nearly 310 nm indicated the presence of a C═S double bond of trithiocarbonate group of DMP-CTA[49,50] and confirmed the formation of B8CYA8-macroCTA. Although the excess DMP-CTA was removed by dialysis, free enzyme was present in the solution in addition to the B8CYA8-macroCTA. The free enzyme was separated by thermal precipitation (vide infra) after polymerization of NIPAM in the presence of B8CYA8-macroCTA.[51]

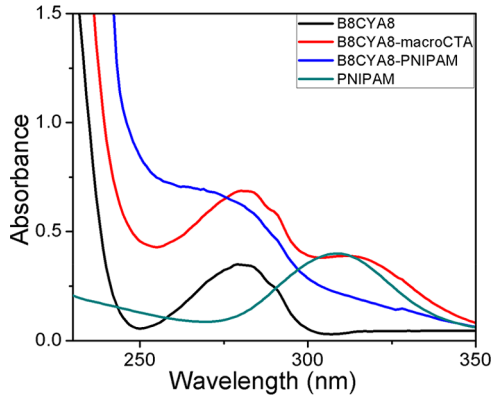

Figure 2. UV−vis spectra of free B8CYA8, B8CYA8-macroCTA, B8CYA8-PNIPAM, and PNIPAM.

Synthesis, Purification, and Characterization of B8CYA8-PNIPAM Conjugate. Next, NIPAM was polymerized using the B8CYA8-macroCTA via RAFT polymerization in the presence of water-soluble VA-044 initiator at 40 °C in phosphate buffer, pH 7.4, at two different [NIPAM]/[B8CYA8-macroCTA]/[VA-044] ratios: 1000/1/1 and 1000/1/2 (Scheme 1). The extent of monomer conversion was determined by ^{1}H NMR spectroscopy (Figure S2). At [NIPAM]/[VA-044] = 1000/1 and initial enzyme concentration of 14 mg/mL, 43% of NIPAM was converted after 24 h. At [NIPAM]/[VA-044] = 1000/2 and initial enzyme concentration of 29 mg/mL during polymerization, 84% conversion was achieved after 4 h only. Using the NMR conversion data, we have determined the theoretical number-average molecular weights ($M_{n,theo}$) by using the following equation: $M_{n,theo}$ = (([NIPAM]/[B8CYA8-macroCTA] × mol wt (MW) of NIPAM × conversion) + (MW of B8CYA8-macroCTA) (summarized in Table S1). We noticed that with increasing [VA-044]/[B8CYA8-macroCTA] ratio and enzyme concentrations, the rate of polymerization increased considerably (Figure S2). The pseudo-first-order kinetic plot for the polymerization of NIPAM, where [NIPAM]/[VA-044] = 1000/1 and initial B8CYA8 concentration was 14 mg/mL is shown in Figure S3. Although it shows around 2 h induction period, which is common for RAFT polymerization, the linearity of the pseudo-first-order kinetic plot indicates a constant concentration of propagating radicals during the course of polymerization reaction.[52] The enzyme-polymer

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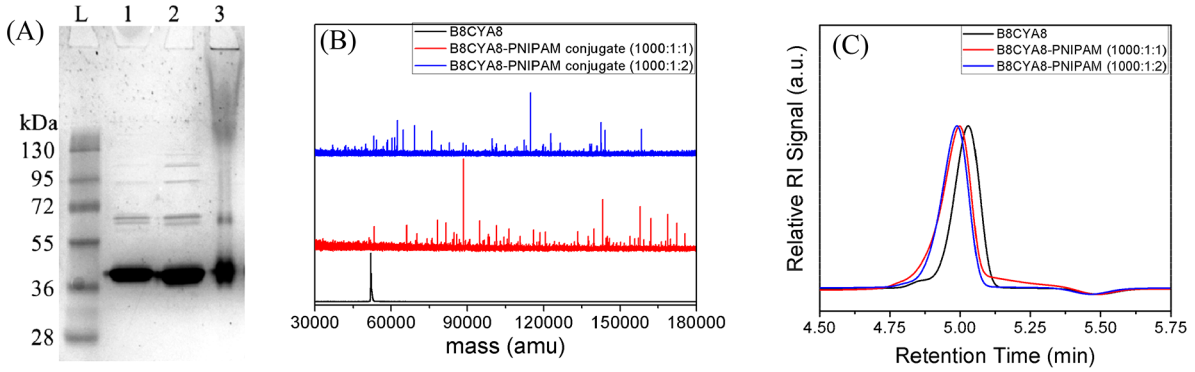

Figure 3. (A) PAGE results of B8CYA8 and B8CYA8-PNIPAM conjugate: Lane L, SDS-PAGE marker; Lane 1, B8CYA8; Lane 2, B8CYA8-macroCTA; Lane 3, B8CYA8-PNIPAM after thermal precipitation; (B) MALDI-TOF spectra for B8CYA8 and B8CYA8-PNIPAM conjugate prepared at two different ratios; and (C) APC RI traces of free B8CYA8 and B8CYA8-PNIPAM conjugated prepared at two different ratios.

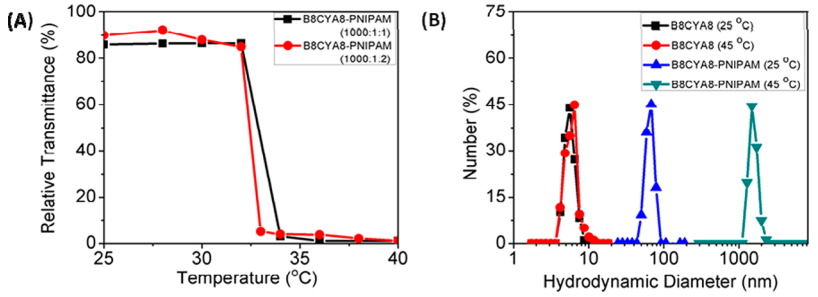

Figure 4. (A) Plot of relative transmittance at 500 nm for the B8CYA8-PNIPAM conjugates vs temperature. (B) Hydrodynamic size distributions as determined by DLS for B8CYA8 and B8CYA8-PNIPAM at 25 and 45 °C.

conjugate (B8CYA8-PNIPAM) was purified through dialysis to remove unreacted monomers and other impurities (but native B8CYA8 was still present in the solution), and initially characterized by ^{1}H NMR spectroscopy (Figure S4). The resonance signal from the polymeric part of the purified conjugate was clearly observed. As protein molecule does not give any peak in NMR,[24] there was no interference from free enzyme in the NMR spectra.

The native enzyme was separated from the conjugate by the thermal precipitation method to obtain pure B8CYA8-PNIPAM conjugate[30] and characterized by polyacrylamide gel electrophoresis (PAGE). The successful formation of the conjugate was verified by PAGE experiment (Figure 3A) showing the native B8CYA8 and B8CYA8-macroCTA, generating identical bands (lanes 1 and 2), and the purified B8CYA8-PNIPAM conjugate (lane 3) as a streak, indicating a distributed mass of the conjugate. The higher molecular weight of the enzyme conjugate with PNIPAM than B8CYA8 suggests a modification of the enzyme through a chemical bond formation since it has been previously reported that PNIPAM or a physical mixture of the polymer and protein does not show such a band distribution on PAGE.[24]

Additional evidence of enzyme modification was obtained from MALDI-TOF mass spectroscopy measurement of B8CYA8 enzyme before and after modification with PNIPAM (Figure 3B). The native B8CYA8 enzyme showed a molecular ion peak at mass-to-charge ratio (m/z) = 52956. After attachment of the polymer, an enhancement of molecular weight and a polymeric mass distribution was observed for both the B8CYA8-PNIPAM conjugates prepared at two different [NIPAM]/[VA-044] ratios.

Furthermore, APC RI traces of these two B8CYA8-PNIPAM conjugates were shifted toward higher molecular weight (lower elution volume) with respect to the free B8CYA8,[53] indicating successful conjugation of the enzyme with PNIPAM (Figure 3C). Number-average molecular weights ($M_{n,APC}$) and $Đ$ values (1.13−1.24) were determined from APC analysis and results are summarized in Table S1. The narrow $Đ$ indicated a good control over molecular weight of the conjugates.

Upon formation of B8CYA8-PNIPAM conjugate, the UV−vis absorption peak at 310 nm was absent (Figure 2). The suppression of this peak could be due to the low C═S concentration in solution arising out of the high degree of polymerization (DP_n) of PNIPAM compared to the B8CYA8-macroCTA. The characterized B8CYA8 absorption signal was clearly observed and indicated enzyme attachment. Only PNIPAM (prepared by RAFT method using DMP as CTA, theoretical number-average molecular weight based on monomer conversion $M_{n,theo}$ = 8000 g/mol) exhibited the C═S double bond characteristic peak at 310 nm from the trithiocarbonate end group in the polymer (Figure 2).

Thermoresponsive Behavior of B8CYA8-PNIPAM Conjugate. Since PNIPAM shows temperature induced phase transition at nearly 32 °C,[54] we studied the thermoresponsive behavior of B8CYA8-PNIPAM conjugate by measuring relative transmittance at different temperatures (Figure 4A). The conjugate showed phase transition (50% reduction of initial transmittance value) at 33 and 32.5 °C for the conjugates prepared at 1000:1:1 and 1000:1:2 ratios, respectively. The thermoresponsive nature of the conjugate was again confirmed by a DLS study (Figure 4B). The native B8CYA8 size at 25 °C is 3.9 nm, whereas the B8CYA8-PNIPAM conjugate at the same temperature is considerably larger (59 nm). At 45 °C,

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which is above the phase transition temperature, the B8CYA8-PNIPAM conjugate is even larger (1060 nm). This considerable enhancement of hydrodynamic diameter above the phase transition temperature is due to the conformational change of PNIPAM from expanded open chain to a globular structure.[55,56] In native B8CYA8, such temperature-dependent changes in diameter were not observed (5.8 nm at 45 °C), as the enzyme probably does not undergo any measurable temperature-dependent conformational change.

Secondary Structure of B8CYA8-PNIPAM Conjugate. The secondary structure of B8CYA8, B8CYA8-macroCTA, and B8CYA8-PNIPAM was interrogated through circular dichroism (CD) measurements (Figure 5). The CD spectrum of native

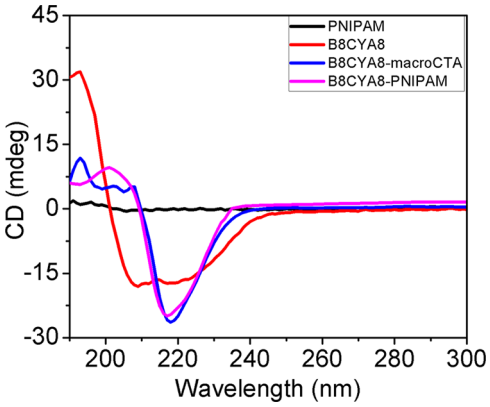

Figure 5. CD spectra of PNIPAM, B8CYA8, B8CYA8-macroCTA, and B8CYA8-PNIPAM conjugate after purification by thermal precipitation.

B8CYA8 shows typical α-helix rich protein secondary structure characteristics with a positive peak at 194 nm and two negative peaks at around 208 and 222 nm. The B8CYA8 crystal structure shows a typical $(\beta/\alpha)_8$ TIM-barrel fold containing both α-helices and β-sheets.[57] The CD spectra of B8CYA8-macroCTA and B8CYA8-PNIPAM conjugate, while similar, show a higher percentage of β-sheet-like structure to indicate structural distortions from native B8CYA8 structure. As expected, PNIPAM by itself did not exhibit any characteristic peak in the CD spectra (Figure 5).[30]

Enzyme Activity and Recycling Potential of B8CYA8-PNIPAM. The half-life ($t_{1/2}$) was determined to measure the kinetic stability of the native enzyme and the enzyme−polymer conjugate (Figure S5). The $t_{1/2}$ of B8CYA8-PNIPAM at 70 °C is more than 80% of that of native B8CYA8. The long $t_{1/2}$ of B8CYA8-PNIPAM at 70 °C indicates thermostability of the conjugate even after incubation at a high temperature. More than 70% of the conjugate is active between 20 to 70 °C, as can be seen in Figure 6A. The conjugate activity is unchanged up to the phase transition temperature (33 °C) and thereafter slightly decreases due to phase separation compared to free enzyme. Nevertheless, the conjugate was kinetically stable and shows that the attachment of the polymer does not have any major effect on enzyme activity in comparison to previous reports of a decrease in activity due to enzyme conjugation or immobilization.[58,59] This is probably due to the higher stability[36] of the native enzyme over a wide range of temperature and retention of the active site structure even after modification. The slight decrease in conjugate activity is probably due to the attachment of the polymer to the reactive thiol group situated near the enzyme active site. Since there is only one thiol group through which attachment occurred, the stability and activity are mostly unaffected.

In a bioreactor, free enzymes cannot be used more than once as recovery from the aqueous solution phase is difficult if not impossible. The B8CYA8-PNIPAM conjugate can be easily separated from aqueous solution above 33 °C and reused for subsequent hydrolysis reactions due to its thermoresponsive nature. To test the recycling potential of this bioconjugate, the recycling assay (see the exact method in the experimental section) was repeated up to four times due to which total product yield increased by 2.4-fold over the free enzyme (Figure 6B). After each cycle of thermal-precipitation, the drop in conjugate activity is due to an approximately 15−20% loss of the conjugate lost from the soluble fraction when decanting the reaction product. After considering this reduction in conjugate amount during each cycle, there was no significant change in activity up to four cycles (Table S2), confirming the advantage of the B8CYA8-PNIPAM over native enzyme and the potential savings when used as part of a cellulase cocktail for biomass.

CONCLUSIONS

In summary, we report a stable B8CYA8-PNIPAM enzyme-polymer bioconjugate. Due to the thermoresponsiveness of the PNIPAM segment, the B8CYA8-PNIPAM conjugate showed temperature-sensitive phase transition at around 33 °C. Enzyme activity is unaltered upon conjugation with PNIPAM below the phase transition temperature of the conjugate and nearly 70% of the conjugate is active up to 70 °C. B8CYA8-PNIPAM bioconjugate recovery and reuse was achieved and is

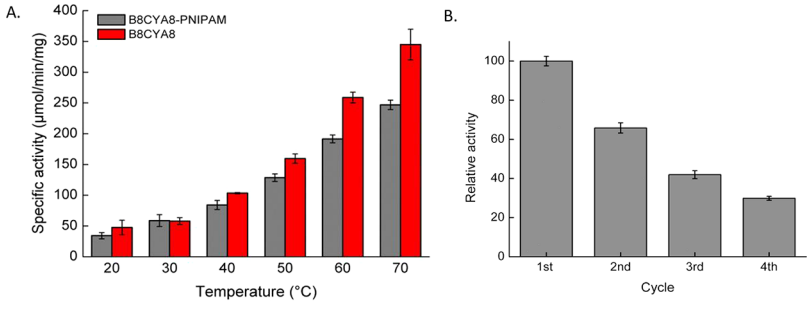

Figure 6. (A) Specific activity of B8CYA8 and B8CYA8-PNIPAM conjugate at 20, 30, 40, 50, 60, and 70 °C. At each temperature, the bar height shows the mean value of three data sets, and the error bar indicates the highest of the three values. (B) Recycling assay for B8CYA8-PNIPAM. The activity of the conjugate was measured at 50 °C after thermal precipitation and the cycle repeated four times. Error bar represents the standard deviation of three replicates.

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a promising target for further optimization toward industrial applications in economical biomass hydrolysis and biofuel production.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.8b00258.

Schematic representation of B8CYA8 showing polymer attachment site, bioconjugate characterization table, RAFT polymerization kinetics of NIPAM in the presence of B8CYA8-macroCTA, and ^{1}H NMR spectra B8CYA8-PNIPAM bioconjugate (PDF).

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Notes

The authors declare no competing financial interest.

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