JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Article
pubs.acs.org/JACS

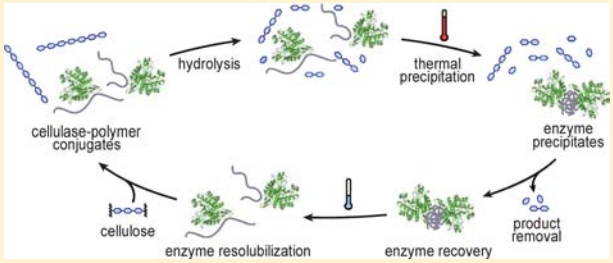
Recyclable Thermoresponsive Polymer–Cellulase Bioconjugates for Biomass Depolymerization

Katherine J. Mackenzie and Matthew B. Francis*

Department of Chemistry, University of California, Berkeley, and Materials Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720-1460, United States

Supporting Information

ABSTRACT: Here we report the construction and characterization of a recoverable, thermoresponsive polymer–endoglucanase bioconjugate that matches the activity of unmodified enzymes on insoluble cellulose substrates. Two copolymers exhibiting a thermoresponsive lower critical solution temperature (LCST) were created through the copolymerization of an aminooxy-bearing methacrylamide with *N*-isopropylacrylamide (NIPAm) or *N*-isopropylmethacrylamide (NIPMa). The aminooxy group provided a handle through which the LCST was adjusted through small-molecule quenching. This allowed materials with LCSTs ranging from 20.9 to 60.5 °C to be readily obtained after polymerization. The thermostable endoglucanase EGPh from the hypothermophilic *Pyrococcus horikoshii* was transaminated with pyridoxal-5′-phosphate to produce a ketone-bearing protein, which was then site-selectively modified through oxime linkage with benzylalkoxyamine or 5 kDa-poly(ethylene glycol)-alkoxyamine. These modified proteins showed activity comparable to the controls when assayed on an insoluble cellulosic substrate. Two polymer bioconjugates were then constructed using transaminated EGPh and the aminooxy-bearing copolymers. After 12 h, both bioconjugates produced an equivalent amount of free reducing sugars as the unmodified control using insoluble cellulose as a substrate. The recycling ability of the NIPAm copolymer–EGPh conjugate was determined through three rounds of activity, maintaining over 60% activity after two cycles of reuse and affording significantly more soluble carbohydrates than unmodified enzyme alone. When assayed on acid-pretreated Miscanthus, this bioconjugate increased the amount of reducing sugars by 2.8-fold over three rounds of activity. The synthetic strategy of this bioconjugate allows the LCST of the material to be changed readily from a common stock of copolymer and the method of attachment is applicable to a variety of proteins, enabling the same approach to be amenable to thermophile-derived cellulases or to the separation of multiple species using polymers with different recovery temperatures.



INTRODUCTION

Lignocellulosic biomass is a highly heterogeneous material composed of lignin, hemicellulose, and cellulose in a complex structure. The ability to convert the cellulose component of this material into fermentable sugars for biofuel production requires the cooperative action of the three cellulase enzymes endoglucanase, exoglucanase, and β-glucosidase. Collectively, these cellulases have been isolated from a broad assortment of organisms and exhibit a range of temperature, pH, and substrate optima.[1] While the exact enzyme cost in the production of lignocellulosic biofuels depends on many factors—including the type of feedstock, enzyme loading, and overall biofuel yield—it is widely recognized that enzyme costs are a significant portion of biofuel prices and pose a key barrier to economically viable fermentation processes.[2,3]

One approach to reducing enzyme costs is to develop methods to collect and reuse enzymes through multiple rounds of processing. With this goal in mind, much work has been done in the field of immobilizing cellulases, including their covalent attachment or adsorption onto substrates such as silicon dioxide wafers,[4] silica,[5] glass beads,[6] calcium alginate beads,[7] and magnetic nanoparticles.[8,9] By rendering the enzymes insoluble, however, access to the crystalline cellulose is potentially impeded and enzyme recovery can be difficult if any insoluble cellulose remains after the maximal hydrolysis yield has been reached. An alternative strategy is the use of stimuli-responsive or "smart" polymers, which are materials that undergo solubility changes in response to external stimuli such as alterations in pH or temperature. There has been some development of reversibly soluble–insoluble polymer–cellulase materials, most commonly utilizing pH-sensitive polymers such as Eudragit L-100 or methacrylic acid polymers.[10−12] These materials limit industrial processes to a fairly narrow pH range and require multiple pH adjustments to recover and reuse the enzyme.

As a more generalizable alternative, one can envision the attachment of cellulases to poly(*N*-isopropylacrylamide) (pNIPAm), which has been well-studied in biotechnology applications because of its thermal responsiveness.[13,14]

Received: September 19, 2012
Published: December 27, 2012

pNIPAm exhibits a highly reversible phase transition at its lower critical solution temperature (LCST) of 32 °C. The polymer is freely soluble below the LCST, but above the LCST the polymer chains undergo a spontaneous coil-to-globule transition, expelling water and precipitating (Figure 1). Several

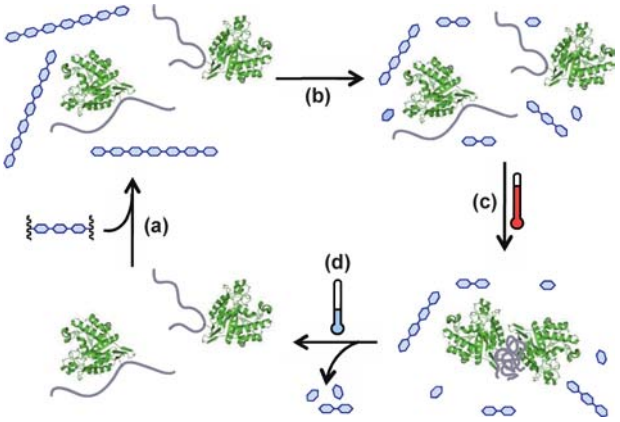


Figure 1. LCST polymer–cellulase activity cycle. The bioconjugate is freely soluble in solution, and then (a) cellulosic substrate is added, (b) the substrate is degraded by the bioconjugate, (c) the temperature of the solution is raised above the LCST to precipitate the polymer–cellulase, and (d) the soluble oligosaccharide product is removed. The temperature of the solution is then decreased below the LCST to resolubilize the bioconjugate, leading back to (a).

groups have utilized pNIPAm to develop a range of thermoresponsive polymer–biomolecule conjugates, typically by attaching the polymers or polymerization initiators to lysine side chains[15] or introduced cysteines.[16] In some cases, small molecule binders such as biotin[17] or glutathione,[18] have been used for protein introduction. As one particularly compelling example, the Hoffman and Stayton groups have conjugated a thermoresponsive polymer to a unique cysteine near the active site of an endoglucanase, with the goal of modulating enzyme activity through polymer collapse above the LCST.[16] They found that the activity could indeed be controlled for the hydrolysis of a soluble substrate simply by changing the reaction temperature. This would suggest that similar LCST-based strategies could lead to recoverable enzymes after cellulose depolymerization, but to date these materials have not been studied for their recycling potential after significant levels of conversion, or for their use with insoluble cellulosic substrates.

Here we report a thermoresponsive endoglucanase bioconjugate that can match and even exceed the activity of unmodified enzymes on insoluble cellulosic substrates. We further demonstrate that this material can be recovered and used for several rounds of cellulose depolymerization, leading to substantially more glucose and cellobiose than can be produced by non-recoverable enzymes. The bioconjugation strategy allows the introduction of the polymer chains at the N-terminus, which is a position that is remotely disposed from the active sites of most cellulases.[19] Finally, the synthetic strategy also allows the LCST of the polymer to be changed readily, enabling the same approach to be amenable to thermophile-derived cellulases or to the separation of multiple species using polymers with different recovery temperatures.

MATERIALS AND METHODS

Unless otherwise noted, all chemicals and solvents used were of analytical grade and were used as received from commercial sources. Room temperature and 4 °C centrifugations were conducted either with a Sorvall RC 5C Plus (Sorvall, USA) for volumes greater than 50 mL, a Sorvall LEGEND Mach 1.6R for volumes between 1 and 50 mL, or an Eppendorf Mini Spin Plus for volumes less than 1 mL (Eppendorf, USA). Centrifugations above room temperature were performed on a Hettich Rotofix 46 H (GMI, Ramsey, MN). Samples were lyophilized using a LAB CONCO Freezone 4.5 (Lab Conco, USA). UV–vis spectroscopic measurements were conducted in a Varian Cary 50 spectrophotometer (Agilent, USA). Fluorescence measurements of 96 well plates were obtained on a SpectraMax M2 (Molecular Devices, Sunnyvale, CA). Tryptophan fluorescence measurements were obtained using a 50 μL quartz cuvette on a Photon Technology International Quanta Master 4 L-format scanning spectrofluorometer (Lawrenceville, NJ) equipped with an LPS-220B 75-W xenon lamp and power supply, A-101013 lamp housing with integrated igniter, switchable 814 photon-counting/analog photomultiplier detection unit and an MD5021 motor driver. Unless otherwise noted, all buffers are 50 mM sodium acetate (NaOAc), pH 4.5.

Synthesis of LCST Copolymers 1a and 1b. The synthesis of *tert*-butyl 2-(3-(2-methylprop-2-enamido)propylamino)-2-oxoethoxycarbamate (MEPO) was adapted from a previously published procedure.[20,21] Azobisisobutylonitrile (AIBN) was recrystallized once from pure methanol (MeOH) and *N*-isopropylacrylamide (NIPAm) was recrystallized twice from hexanes and toluene before use. Polymerization was conducted using a 1:9 molar ratio of MEPO:NIPAm and weight percent 11.1:0.6:88.3 for monomers:AIBN:CH_3OH. MEPO (378.5 mg, 1.2 mmol), NIPAm (1.22 g, 10.78 mmol), and AIBN (80 mg, 0.49 mmol) were added to a clean scintillation vial. The vial was purged and refilled N_2. MeOH (12.67 g, 395.4 mmol) which had been previously sparged with N_2 for 1 h was added, and the components were dissolved under N_2. The mixture was divided into six clean scintillation vials, a stream of N_2 was bubbled through the solution in each vial for 10 min, and the vials were sealed under N_2 and placed in a 60 °C oil bath for 6 h. The polymer was recovered from the reaction mixture by one precipitation from MeOH into cold diethyl ether followed by centrifugation. It was dissolved in 1:1 CH_2Cl_2:trifluoroacetic acid for 1 h, concentrated in vacuo, and then neutralized using 5 M NaOH. The polymer was purified through ultrafiltration (10 kDa molecular weight cutoff [MWCO]) and lyophilized to afford the final poly(MEPO-*co*-NIPAm) (**1a**). The molar ratio of MEPO:NIPAm was calculated to be 1:10.3, or 8.6% incorporation of MEPO, through ^{1}H NMR analysis (see Figure S1). SEC analysis using PMMA standards indicated M_n = 85 127, M_w = 155 321, and PDI = 1.82. The same general procedure was followed to make a copolymer with MEPO and *N*-isopropylmethacrylamide (NIPMa) (**1b**). From analysis of the ^{1}H NMR spectrum, the molar ratio of MEPO:NIPMa was calculated to be 1:10.36, or 8.8% incorporation of MEPO (see Figure S2). SEC analysis using PMMA standards indicated M_n = 11 144, M_w = 14 037, and PDI = 1.26.

Small-Molecule Modification of 1a and 1b. A 20 mg/mL stock solution of 1a was made in pH 4.5 buffer. A series of 1 mL, 120 mM stock solutions of formaldehyde, acetone, 3-fluoroisonicotinaldehyde (with 10% DMSO), and 4-hydroxy-2-butanone, and 1.2 M stock solutions of D-(+)-mannose and D-(+)-dextrose were made in pH 4.5 buffer. Next, 750 μL of the polymer solution was mixed 1:1 with each of the small molecule solutions in 4 mL glass dram vials. The reactions were incubated at rt for 24 h, and then excess small molecules were removed and the polymers buffer exchanged into pure water through eight rounds of ultrafiltration (10 kDa MWCO) at 4 °C. They were lyophilized and analyzed by NMR spectrometry to confirm modification, and the LCST was determined. The same procedure was followed for small-molecule modification of **1b**.

LCST Measurements. Polymer samples were dissolved in pH 4.5 buffer at a concentration of 1 mg/mL and mixed for 30 min to ensure complete dissolution. They were transferred to a cuvette with a stir bar

dx.doi.org/10.1021/ja309277v | J. Am. Chem. Soc. 2013, 135, 293–300

and warmed at a rate of 0.5 °C/min while stirring in a Horiba Scientific F-3004 Peltier device (Kyoto, Japan) controlled by a LFI3751 5A digital temperature control instrument (Wavelength Electronics, Bozeman, MT). The cuvettes were quickly removed every 0.5 °C, the absorbance at 600 nm was measured, and then the cuvette was returned to the Peltier device. The LCSTs reported here are the temperature at 10% of the maximum absorbance for each sample.

Expression and Purification of AKT-EGPh. BL21starDE3 *E. coli* cells containing an EGPh-pet24b plasmid were obtained from the Douglas Clark laboratory at UC Berkeley.[22] The plasmid initially contained both N-terminal and C-terminal His_6 tags, so site-directed mutagenesis and restriction digestion were used to remove the N-terminal His_6 tag and to install an AKT sequence at the N-terminus to maximize transamination yield (see Supporting Information). AKT-EGPh plasmids were transformed into One Shot BL21 (DE3) *E. coli* cells (Invitrogen) via heat shock and plated on Luria broth (LB) agar plates containing kanamycin (50 μg/mL). Cultures were grown in 1 L of LB containing kanamycin (50 μg/mL) at 37 °C until an optical density (OD) of 0.5 was observed at 600 nm. Expression of AKT-EGPh was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were grown for 12 h at 25 °C and then spun down at 7000 rcf, 4 °C for 40 min to pellet the cells. The cells were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose resin following the recommended protocol (Qiagen). The purified protein was buffer exchanged into 50 mM NaOAc buffer (pH 4.5) through ultrafiltration (10 kDa MWCO) to yield 85 mg of purified protein per L of culture.

Small-Molecule Modification of EGPh. Transamination of the EGPh N-terminus was performed following a previously reported method.[23] EGPh at a concentration of 50–60 μM in pH 4.5 buffer was mixed 1:1 with a pH 4.5 solution of 200 mM pyridoxal-5′-phosphate (PLP). Samples were reacted for 1 h at 37 °C, and then excess PLP was removed by eight rounds of ultrafiltration (30 kDa MWCO) at 4 °C. Controls were conducted following the same procedure but without PLP. To modify the transaminated protein with benzylalkoxyamine ($BnONH_2$), 125 μL of a 250 mM solution of $BnONH_2$ (pH adjusted to 5.5) was added to 625 μL of transaminated EGPh (30 μM) in pH 4.5 buffer and incubated at rt for 42 h. Excess $BnONH_2$ was removed through ultrafiltration (30 kDa MWCO). A control was conducted following the same procedure but with non-transaminated EGPh. A portion of both samples were submitted for LC-MS analysis to determine the level of modification achieved (Figure S3). To modify transaminated protein with PEG, a stock solution of 1 mM 5 kDa alkoxyamine-poly(ethylene glycol) (PEG)[24] in pH 4.5 buffer was mixed 1:1 with 50 μM transaminated EGPh and incubated at rt for 42 h. Excess PEG was removed through ultrafiltration (30 kDa MWCO). A control was conducted following the same procedure but with non-transaminated EGPh. A portion of both samples were visualized by SDS-PAGE (see Figure 3).

Construction of EGPh–Polymer Bioconjugates. One mL of a 40 mg/mL stock solution of 1a in pH 4.5 buffer was combined with 1 mL of 50 μM transaminated EGPh in a 4 mL glass dram vial and pipetted vigorously to mix. The solution was incubated at rt for 24 h, and then 2 mL of 1.2 M D-(+)-mannose in pH 4.5 buffer was added, mixed via pipet, and the new mixture was incubated an additional 24 h at rt. The solution was transferred to a 15 mL Falcon tube and heated for 10 min in a 55 °C water bath to precipitate the polymer, and then centrifuged for 5 min at 55 °C and 2000 rpm. The supernatant was removed, and the pelleted polymer was resuspended in the same volume of rt buffer (pH 4.5). This procedure was repeated three more times for a total of four precipitation cycles, and then any excess mannose was removed through three cycles of ultrafiltration at 4 °C (10 kDa MWCO). The concentrated polymer–EGPh conjugate was transferred to an Eppendorf tube and stored at 4 °C. A small portion of the purified conjugate was buffer exchanged into pure water using ultrafiltration (30 kDa MWCO), lyophilized, and analyzed for protein concentration using tryptophan fluorescence. Attachment of **1b** was performed using a similar procedure. A control with **1a** was conducted following a similar procedure but with non-transaminated EGPh.

Protein Quantification. Unmodified, transaminated, PEGylated, and $BnONH_2$-modified EGPh concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific), with an extinction coefficient of 139 020 M^{-1} cm^{-1} and molecular weight of 49 023 Da. Protein concentration of the polymer conjugates was determined using tryptophan fluorescence. Buffered standards at pH 4.5 were prepared in triplicate containing 5 mg/mL of mannose-quenched copolymer and 7.06, 5.01, 3.0, 1.0, and 0 μM EGPh. Triplicate 5 mg/mL samples of lyophilized polymer–EGPh conjugate were made in pH 4.5 buffer. The fluorescence spectrum of each of the standard and experimental samples was collected from 290 to 400 nm, with excitation at 280 nm. The maximum fluorescence intensity of each standard set was plotted versus the protein concentration and a linear fit was applied to the data points (all $R^2 > 0.99$). This linear fit was used to calculate the protein concentration in the lyophilized samples using their fluorescence maxima. Serial dilutions of the reserved, non-lyophilized protein–polymer conjugate were prepared and their fluorescence intensities were measured to determine the dilution level that matched that of the 5 mg/mL lyophilized samples. From these data, the protein and polymer concentrations of the reserved polymer–EGPh conjugates were determined. The protein concentrations in μM EGPh/mg material were 0.520 μM/mg for **1a**–EGPh, 0.177 μM/mg for **1b**–EGPh, and 0.066 μM/mg for a control of **1a** combined with non-transaminated EGPh.

Activity of Modified EGPh. All protein samples were assayed in triplicate in 1.5 mL Eppendorf tubes containing stir bars at 40 °C, using a 1% (w/v) suspension of Sigmacell cellulose powder (Sigma-Aldrich) in pH 4.5 buffer and 0.2 μM protein. Additional mannose-quenched **1a** or **1b** was added to the polymer–EGPh bioconjugate assays to a total concentration of 2 mg/mL polymer. To measure the reactions, each tube was shaken vigorously to ensure even distribution of the substrate and protein and a 100 μL aliquot was immediately removed and transferred to a clean, empty Eppendorf tube. This aliquot was centrifuged for 1 min at 13.2k rpm, and then the clarified supernatant was transferred to a 0.6 mL Eppendorf tube and immediately frozen in dry ice. The supernatant aliquots were stored at −20 °C until analysis for the amount of soluble reducing sugar.

Activity of Recycled Polymer–EGPh Conjugates. In both recycling assays, a 100 μL aliquot was taken at $t = 0$, and a 50 μL aliquot was removed at the end of 12 h to measure the amount of reducing sugar. The stir bars were removed, and the polymer-containing tubes were heated at 55 °C for 5 min to precipitate the polymer and then centrifuged at 2k rpm for 10 min at 55 °C to pellet the polymer. All tubes were then centrifuged at 13.2k rpm for 1 min at rt to pellet the substrate. The cleared supernatant was removed and replaced with ice cold buffer, clean stir bars were added, and the tubes shaken vigorously for 3 min to ensure an even suspension of substrate and polymer. The assay procedure was then repeated twice more, beginning with removing a 100 μL aliquot to measure initial reducing sugar, for a total of two precipitation events and three 12 h assay periods.

Acid and steam-pretreated Miscanthus (*Miscanthus giganteus*) was obtained from the laboratory of Prof. Douglas Clark at UC Berkeley. The substrate had been cut into approximately 1-in. pieces and then subjected to 1.5% (w/w) sulfuric acid, 25% biomass loading (w/w), at 190 °C for approximately 1 min. It underwent a steam explosion step, and then the solids were pressed to remove liquids. It was then washed extensively with deionized water until the filtrate had a neutral pH and no detectable glucose. The material was dried for 24 h at 104 °C and then ground into a fine powder with a mortar and pestle.

Analysis of Soluble Reducing Sugar. This procedure was performed following a previously reported method, using the glucose oxidase–peroxidase assay with OxiRed as the substrate.[22] Analysis was performed in clear-bottom plastic 96-well plates, with each sample analyzed in triplicate. Internal standards of 300, 200, 100, 50, 25, and 0 μM glucose, and 150, 100, 50, 25, and 12.5 μM cellobiose in pH 4.5 buffer were included in each plate. Frozen aliquots from the activity assays were thawed on ice and then diluted 0- to 20-fold with cold buffer, and then 8 μL of the solution was incubated with 8 μL of β-glucosidase (5 mg/mL in 10 mM NaOAc pH 4.6) for 60 min at 37 °C

to convert all of the cellobiose to glucose. The amount of glucose present was then measured by adding 65 μL of glucose oxidase (1.25 U/mL), horseradish peroxidase (1.25 U/mL), and OxiRed (60 μM) in 125 mM phosphate buffer (pH 7.45) and incubating at rt for 10 min in the dark. The amount of Resorufin formed was measured on an optical plate reader with excitation at 535 nm and emission detection at 590 nm. The amount of Resorufin formed corresponded to the amount of glucose present. Linear standard curves were made from the internal standards in each plate (all $R^2 > 0.97$), which were then used to calculate the amount of glucose equivalents present in each activity assay sample. The triplicate measurements of each supernatant sample were averaged, and then the measurements of the triplicate activity assay samples were averaged to calculate each data point.

RESULTS AND DISCUSSION

The key synthetic requirement for these studies is the site-selective attachment of LCST polymers to cellulase enzymes. Although the attachment of polymer chains is a common bioconjugation practice, there are relatively few strategies for doing so that are site-specific. In previous work, we have reported the attachment of PEG chains to ketone and aldehyde groups on protein surfaces through oxime formation.[24] This method provides a hydrolytically stable linkage and can be formed under mild pH and temperature conditions. We have also used this strategy to incorporate protein cross-links into methacryl hydrogels,[20,21] and other laboratories have used it to immobilize proteins on polymer films.[25] To introduce multiple copies of the requisite aminooxy handles into the temperature-responsive polymer, free-radical copolymerizations were performed with a Boc-aminooxy methacrylamide and *N*-isopropylacrylamide (NIPAm) (yielding **1a**) or *N*-isopropylmethacrylamide (NIPMa) (yielding **1b**) using AIBN as a radical initiator (Figure 2a). A 1:9 starting molar ratio of aminooxy monomer:NIPAm or NIPMa was used in methanol at 60 °C for 6 h. Following ether precipitation, the copolymers were deprotected with trifluoroacetic acid in dichloromethane. Characterization by ^{1}H NMR spectroscopy showed an aminooxy monomer incorporation of 8.6% for **1a** and 8.8% for **1b** (Supporting Information Figures S1 and S2). Polymer size was determined by size exclusion chromatography (SEC) using poly(methyl methacrylate) standards, with number-average molecular weights (M_n) of 85 127 and 11 144 Da and polydispersity indices (PDI) of 1.82 and 1.26 for **1a** and **1b**, respectively. Polymer that was subjected to thermal precipitation, isolated by filtration, and redissolved provided identical ^{1}H NMR spectra, suggesting that the aminooxy monomers were incorporated throughout the material.

A well-studied phenomenon of LCST polymers is the ability to adjust the thermoprecipitation point by changing the hydrophilicity of the material.[26,27] To determine the LCST of **1a** and **1b**, 1 mg/mL polymer samples in sodium acetate buffer (50 mM, pH 4.5) were warmed at a rate of 0.5 °C/min while stirring, and the absorbance at 600 nm was measured every 0.5 °C. Incorporation of the hydrophilic aminooxy monomomer increased the LCST of the pNIPAm copolymer to 42.5 °C from 32 °C for the homopolymer, and the LCST of the pNIPMa copolymer increased to 58.1 °C from 43 °C.[28]

In addition to providing a potential method for protein attachment, the aminooxy group also provides a handle through which the LCST can be adjusted through small-molecule quenching (Figure 2). This allows a wide range of LCSTs to be accessed starting from a common supply of copolymer. Quenching also prevents the aminooxy functional groups from reacting with adventitious aldehydes, such as those of the

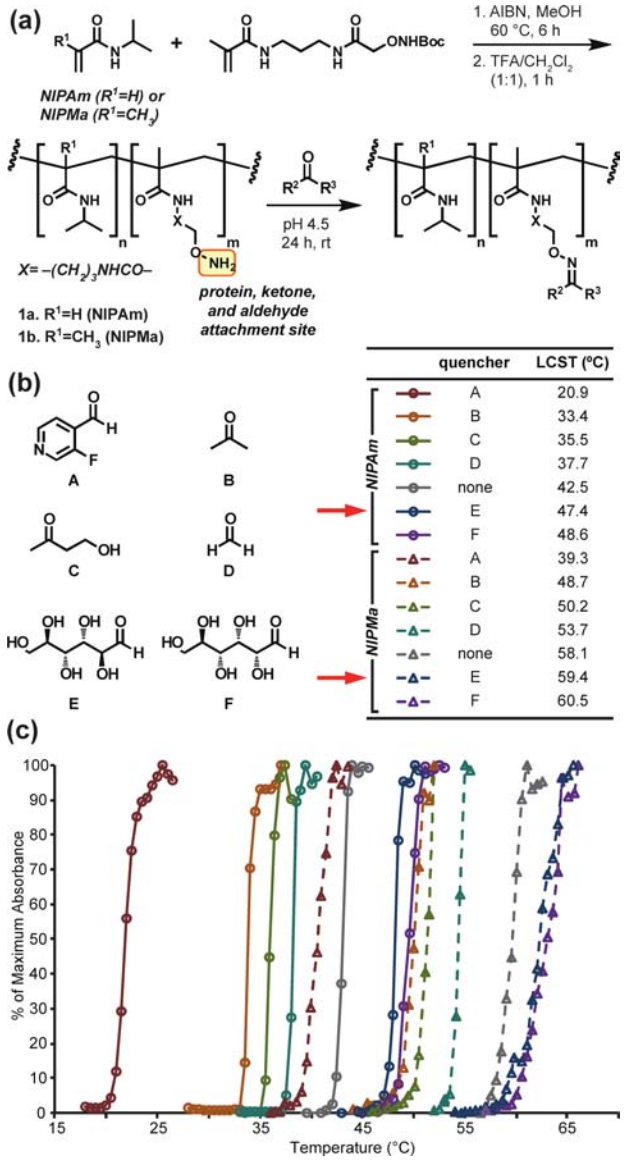


Figure 2. Construction and LCST tuning of copolymers. (a) A Boc-aminooxy methacrylamide was copolymerized with either NIPAm or NIPMa to make thermoresponsive copolymers **1a** and **1b**, respectively. (b) Deprotected copolymers were reacted with 60 mM of small molecules A, B, C, or D, or 600 mM of E or F for 24 h at pH 4.5. LCSTs reported are the temperature at 10% of maximum absorbance at 600 nm. (c) Graphs of the LCST measurements, normalized to % of maximum absorbance. Red arrows indicate the quencher chosen to construct the EGPh–polymer bioconjugates.

glucose molecules produced during cellulose depolymerization. A 10 mg/mL solution of **1a** or **1b** was reacted with six different small molecules (60 or 600 mM) for 24 h, and then purified by ultrafiltration and lyophilized. Using this strategy and starting from only two copolymers, materials were obtained with LCSTs ranging from 20.9 to 60.5 °C (Figure 2c). From these possibilities, we decided to use mannose to quench the protein–polymer bioconjugates because of its economical cost, low likelihood to affect enzyme activity, and compatibility with our method of glucose quantification in further experiments.

Following the construction of LCST copolymers with tunable precipitation temperatures, we next focused on the modification of the enzyme. We chose to use a hyper-

dx.doi.org/10.1021/ja309277v | J. Am. Chem. Soc. 2013, 135, 293–300

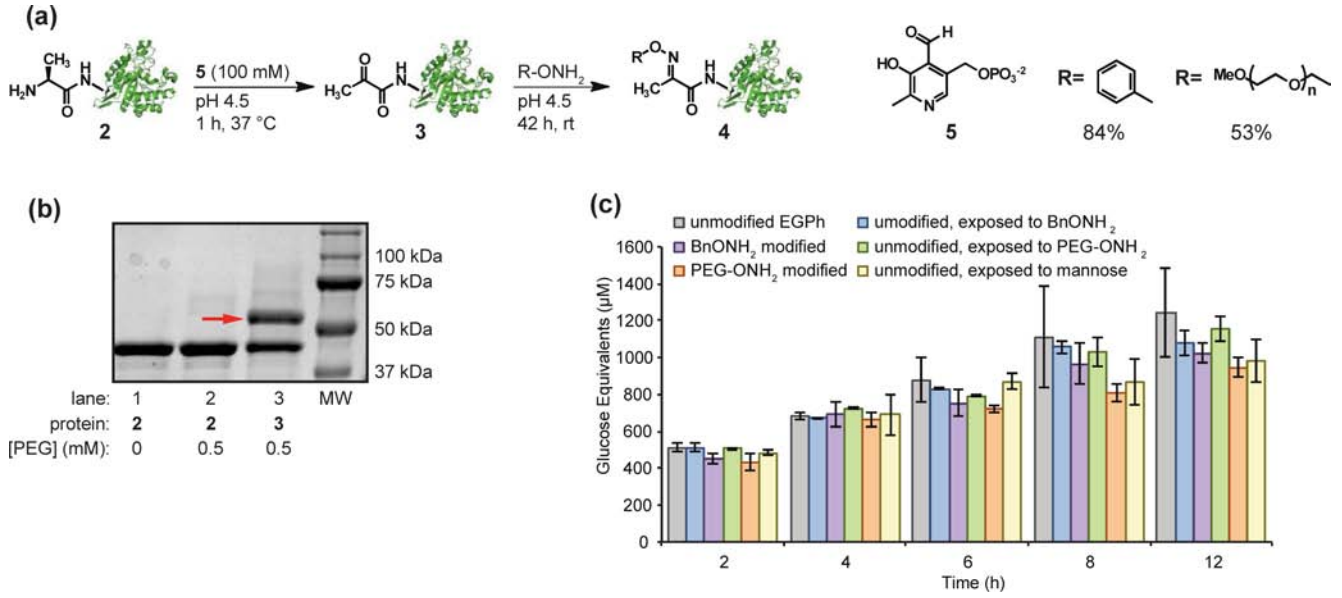


Figure 3. Assessing protein modification. (a) Purified EGPh (**2**) was reacted with **5** to yield **3**, and then modified with benzylalkoxyamine or 5k-PEG-ONH_2. Modification yields were obtained by LC-MS analysis (Figure S3) or (b) by SDS-PAGE analysis followed by Coomassie staining and densitometery. The red arrow indicates a 5 kDa increase in mass corresponding to a single covalent modification with 5k-PEG-ONH_2. (c) Modified and unmodified protein samples were assayed for hydrolytic activity. The error bars represent the standard deviation of three replicate experiments.

thermophilic endoglucanase from the deep-sea archaeon *Pyrococcus horikoshii* (EGPh). This family 5 cellulase was discovered in 2002, and its ability to hydrolyze a variety of cellulosic substrates and stability at temperatures above 97 °C makes it a promising candidate for industrial applications.[22,29] Our method of protein modification involved a previously reported site-selective transamination, in which pyridoxal 5′-phosphate (PLP) is used to oxidize the N-terminus of proteins to yield a ketone group (Figure 3a). In a subsequent step, the N-terminus can be modified selectively through oxime formation with aminooxy-functionalized small molecules or the aminooxy-substituted LCST polymers described above.[30,31]

We have previously identified that installing an alanine-lysine motif at the N-terminus of proteins leads to optimal transamination levels.[23] An ala-lys-thr sequence was inserted at the N-terminus of EGPh and the new construct was expressed in *Escherichia coli*. Using a C-terminal His_6 tag for purification with Ni-NTA chromatography, an average yield of 85 mg of purified protein per L of expression media was obtained (Figure 3b, lane 1). The N-terminus of the protein was then site-selectively transaminated by reacting with PLP (100 mM) for 1 h. To establish the level of transamination and confirm the site-selectivity of the reaction, the transaminated protein was incubated with benzylalkoxyamine (42 mM, pH 4.5) or 5k-aminooxy-poly(ethylene glycol) (PEG)[24] (500 μM, pH 4.5) for 42 h, followed by ultrafiltration. Samples were analyzed by LC-MS to determine a small-molecule modification yield of 84%, and SDS-PAGE followed by Coomassie staining and densitometry showed modification with a single PEG chain in 53% yield (Figure 3b and Supporting Information Figure S3).

Next, assays were performed to ascertain whether the enzyme modification site or the conditions used negatively affected its catalytic activity. Each sample was evaluated in triplicate, using 0.2 μM EGPh and an insoluble cellulose substrate (Sigmacell, 1% w/v). Both benzylalkoxyamine and 5 kDa-PEG-alkoxyamine modified EGPh were assayed, along with two controls in which the protein was not transaminated but was still incubated with the alkoxyamines for 42 h. These controls showed no modification by LC-MS and SDS-PAGE analysis (Figure 3b, lane 2). In addition, an unmodified, non-transaminated control was included, as well as a sample of EGPh that had been incubated with mannose (600 mM) for 24 h to ensure protein activity would not be affected by the quenching step used for the LCST polymers. Each protein reaction was assayed in a 40 °C water bath, and 100 μL aliquots were removed at 2, 4, 6, 8, and 12 h. The supernatants were analyzed for the amount of soluble sugar released using the glucose oxidase−peroxidase assay with OxiRed as the fluorescent substrate.[22]

As shown in Figure 3c, the activities of the two modified endoglucanases were slightly reduced relative to the unmodified control, but the differences were generally within the standard deviations of the assays. The activities of unmodified enzyme samples that had been exposed to benzylalkyoxyamine, PEG-alkoxyamine, and mannose were also slightly lower, but within one standard deviation of the activity of the unmodified control at each time point. From these experiments, we concluded that neither the N-terminal modification itself nor the reaction conditions used had a significant effect on endocellulase activity.

To generate the thermoresponsive material, ketone-bearing EGPh was covalently attached to the aminooxy-substituted LCST polymers. Transaminated EGPh was combined with polymer **1a** or **1b** and allowed to react at room temperature for 24 h. Any remaining aminooxy groups on the polymers were then capped by the addition of mannose (600 mM), followed by further incubation at room temperature for 24 h. To remove any unmodified protein and free mannose, the mixture was heated to 7 °C above the LCST and centrifuged at that temperature to pellet the precipitated polymer bioconjugate, and then the supernatant was removed and replaced. This was repeated for a total of four cycles. To determine the amount of protein that could be non-covalently adsorbed onto the

dx.doi.org/10.1021/ja309277v | J. Am. Chem. Soc. 2013, 135, 293−300

polymer, this same procedure was also performed using non-transaminated EGPh and polymer **1a**. A small portion of each material was then lyophilized to measure protein attachment.

The protein−polymer ratio was determined by measuring the tryptophan fluorescence of the weighed lyophilized sample compared to standards that contained mannose-quenched **1a** or **1b** and EGPh. This analysis indicated protein:polymer ratios of 25.5 mg/g for the NIPAm bioconjugate (corresponding to 0.044 protein/polymer chain) and 8.7 mg/g for the NIPMa bioconjugate (corresponding to 0.002 protein/polymer chain). The NIPAm copolymer + unmodified EGPh control had a ratio of 3.2 mg/g. For enzyme activity experiments, serial dilutions of the reserved, non-lyophilized protein−polymer conjugates were analyzed to find the dilution levels with fluorescence values that matched those of the lyophilized samples.

To assess any change in activity as a result of polymer attachment, an activity assay was performed using the unmodified, non-transaminated EGPh, the **1a**−EGPh conjugate, and the **1b**−EGPh conjugate. Additional mannose-quenched **1a** or **1b** was added to the **1a**−EGPh and **1b**−EGPh assays to bring the total polymer concentrations up to 2 mg/mL, so the conditions would be comparable to subsequent recycling experiments in which additional polymer was added to enhance bioconjugate aggregation. It was observed that both **1a**−EGPh and **1b**−EGPh displayed about half the endoglucanase activity of the free EGPh after 2 h, but at later time points the enzymatic activity in the samples converged (Figure 4a). After 12 h, any initial differences in activity had subsided and the differences in the total concentration of reducing sugars was statistically insignificant. Other studies of endogluconases have observed similar decreases in activity over time. The mechanisms for this inactivation are complex, and could involve product inhibition, protein adsorption on the substrate, and denaturation.[32−34] In the case of EGPh, we have found that product inhibition by cellobiose is unlikely to be the cause of this activity loss, as added beta-glucosidase does not lead to increases in the overall activity of the unmodified enzyme (as measured at 12 h, Supporting Information Figure S4). Regardless of the cause, this deactivation places an upper boundary on the total amount of product that can be obtained, allowing the polymer−enzyme conjugates to reach similar conversion levels.

The activity of the polymer bioconjugates was equivalent to free protein after 12 h, but if the materials could be precipitated, collected, and reused, the total amount of reducing sugar produced over the lifetime of the material would potentially be much greater than that possible using only free enzyme. To test the recycling potential of the bioconjugates, a free unmodified EGPh control and the **1a**−EGPh bioconjugate were prepared as described for the previous assay and allowed to react for 12 h at 40 °C. The bioconjugate was then heated to precipitate the protein−polymer material and centrifuged to pellet the aggregated polymer and the cellulosic substrate. The EGPh control was also centrifuged. The supernatant of all samples was removed and replaced with an equal volume of fresh, ice-cold buffer to ensure rapid bioconjugate resolubilization, and the mixtures were again allowed to react for 12 h at 40 °C. This was repeated once more, for a total of two precipitations and three cycles. Aliquots of each reaction were removed at the beginning and end of each cycle to measure the concentration of additional reducing sugar produced during each 12 h.

As shown in Figure 4b, after the initial 12 h of reaction, the **1a**−EGPh bioconjugate produced reducing sugars at 86% of the level of the control. However, it retained 68% and 63% of its initial activity over two cycles of precipitation and recovery of the material. The free enzyme, in contrast, retained only 11% and 4% of its initial activity. While the supernatant was removed from all samples, only the polymer bioconjugate sample had the majority of the protein precipitated in the substrate pellet. Any subsequent activity for the free control was due to enzyme carryover contained in the pelleted insoluble cellulose. Over three cycles, the polymer−protein bioconjugate was able to produce a 1.7-fold increase in the amount of free reducing sugar over that produced by the control (Figure 4c).

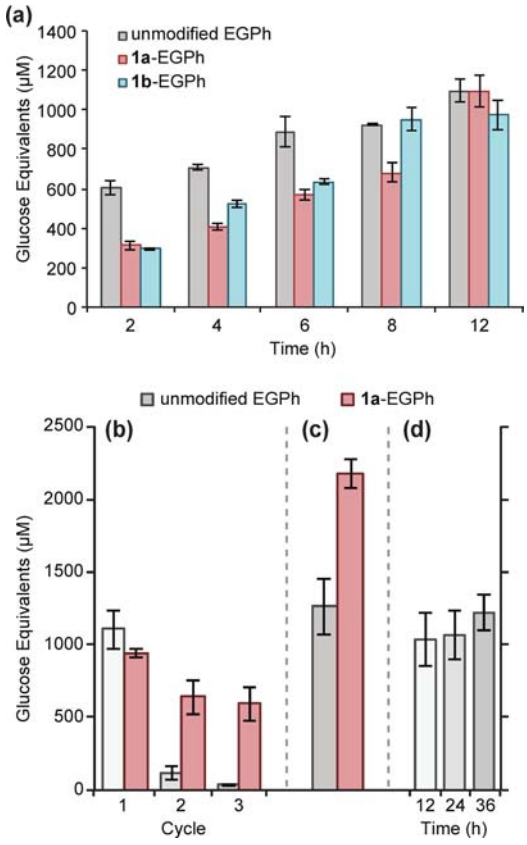


Figure 4. Hydrolytic activity of polymer−cellulase bioconjugates on Sigmacell. Error bars represent the standard deviation of three replicate experiments for all graphs. (a) Activity of bioconjugates **1a**−EGPh and **1b**−EGPh. (b) Recycling assay for **1a**−EGPh. Soluble reducing sugar was measured at 0 and 12 h of each cycle; the difference is shown here. (c) Total additional glucose equivalents produced over three cycles. Shown here is the sum of the values from (b). (d) Hydrolytic activity of unmodified EGPh over 36 h. Shown is the difference in soluble reducing sugar between 0 h and 12, 24, and 36 h.

While it is clear the polymer can be recycled, we also wanted to compare its activity over three 12 h reaction cycles to the activity of free protein over one 36 h reaction to determine if recycling the enzyme actually provided any benefit over simply letting free enzyme react for an extended period of time. Free, unmodified EGPh was allowed to react with Sigmacell for 36 h at 40 °C, with free reducing sugar measured at 0, 12, 24, and 36 h. Consistent with the results above, the free enzyme reached an upper activity limit by 12 h, with only modest increases in reducing sugar achieved in the next 24 h (Figure 4d). At 12 h, the polymer−protein bioconjugate was 91% as active as the free control; by recycling the enzyme, however, 78% more reducing

dx.doi.org/10.1021/ja309277v | J. Am. Chem. Soc. 2013, 135, 293−300

sugars were produced over 36 h than if the free enzyme was allowed to react.

We next decided to characterize the bioconjugate using a more realistic substrate that included hemicellulose and lignin. We chose the perennial grass Miscanthus (*Miscanthus giganteus*), as this plant has been extensively studied for its potential as a large scale feedstock for biofuels production.[36] Acid- and steam-pretreated Miscanthus was washed, dried, and ground into a powder. The activity and recycling ability of the **1a**–EGPh bioconjugate was then assessed following the same procedure that was used for Sigmacell. As seen in Figure 5, the

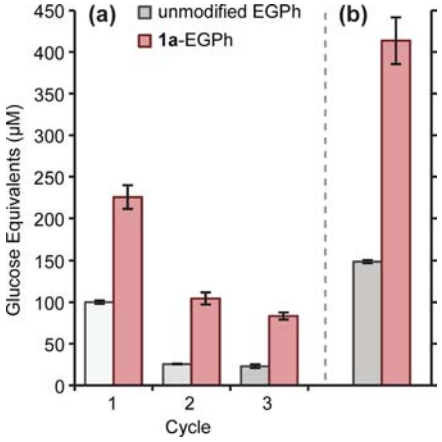


Figure 5. Hydrolytic activity of **1a**–EGPh on Miscanthus. Error bars represent the standard deviation of three replicate experiments for all graphs. (a) Recycling assay for **1a**–EGPh. Soluble reducing sugar was measured at 0 and 12 h of each cycle; the difference is shown here. (b) Total additional glucose equivalents produced over three cycles. Shown here is the sum of the values from (a).

activity of the unmodified enzyme control was significantly lower for Miscanthus that it was for Sigmacell. This drop is not unexpected, as Miscanthus has much greater chemical and structural heterogeneity than substrates consisting of isolated cellulose. In addition, protein adsorption onto lignin and subsequent deactivation is a known challenge with unrefined biomass.[22,34,35] The activity of the bioconjugate also decreased for this substrate, but to a lower extent than the control. At the same enzyme loading, the bioconjugate produced substantially more reducing sugars after the first cycle. This activity difference between the bioconjugate and the control on Miscanthus is similar to that seen upon addition of certain surfactants and polymers to the enzymatic hydrolysis of lignocellulic biomass.[34,35] Specifically, the addition of surfactants such as Tween and Tiron, or polymers such as PEG, has been shown to increase saccharification of lignocellulosic biomass (albeit only by increases of <20% in most cases).[34,36] The mechanisms behind these observations have not been completely elucidated, but common explanations invoke interactions between the additives and lignin that prevent enzyme adsorption onto the hydrophobic surface or enhance its subsequent desorption. Either mechanism would increase the concentration of active enzyme in solution. It appears that the polymers in this study also shows this effect.

In subsequent cycles of reuse, there was a drop in activity of the bionconjugate, presumably because some protein is still adsorbed onto the biomass and ultimately deactivated. However, the bioconjugate remained 104% and 83% as active as the initial control in cycles 2 and 3. The ability to recover the bioconjugate, combined with the added activity due to the surfactant effect, increased the amount of reducing sugars by 2.8-fold over three rounds. We are currently investigating the mechanism through which the activity enhancement occurs, and we are further optimizing the polymer component to minimize adsorption further.

■ CONCLUSION

Through these studies, highly adaptable thermoresponsive polymer–protein bioconjugates have been developed through the copolymerization of NIPAm or NIPMa with an alkoxyamine-bearing methacrylamide. The two copolymers exhibited LCSTs of 42.5 and 58.1 °C, respectively, but small-molecule quenching of the alkoxyamine pendant groups of those two copolymers allowed materials with LCSTs ranging from 20.9 to 60.5 °C to be obtained. To allow polymer attachment, the hyperthermophilic endoglucanase from *Pyrococcus horikoshii* (EGPh) was site-selectively transaminated using pyridoxal 5′-phosphate. Compared to free enzyme, protein modified with the small molecule benzylalkoxyamine or the polymer 5 kDa-PEG-alkoxyamine showed insignificant decreases in producing soluble reducing sugars from hydrolysis of an insoluble cellulosic substrate after 12 h. Protein modified with either of the two NIPAm or NIPMa copolymers exhibited a decrease in activity initially, but the levels of soluble reducing sugars were comparable to those of the unmodified control protein after 12 h. The NIPAm copolymer–protein conjugate retained over 60% of its initial activity following two cycles of thermal recovery, and a total of 1.7-fold more soluble reducing sugars were produced over three cycles compared to the unmodified control. When applied to a sample of Miscanthus, the enzyme bioconjugate provided both an increase in overall activity and the capability of recycling to result in a 2.8-fold increase in depolymerized product.

In this way, a method for the recovery and reuse of cellulase enzymes was achieved that could be applied to the wide range of cellulases currently being studied for industrial applications. In addition, the polymer properties could be adjusted to be compatible with the different temperatures at which these enzymes are used. It is difficult to obtain cost estimates of cellulase enzymes on industrial scale, as research in biomass depolymerization is rapidly developing.[2] However, we currently estimate that both the enzymes and the LCST polymers can be obtained on bulk scale for $50/kg or less. When the advantages of recycling are taken into account, these figures suggest that this concept could be economically feasible if a 1:1 ratio of protein to polymer can be used. While we consider the bioconjugation methods used herein to be practical and inexpensive, our current bioconjugate loadings are admittedly far below this number due to the high concentrations of functional groups that are required for oxime formation. Unfortunately, there are currently few alternative strategies for the site-specific, stoichiometric, and scalable attachment of polymers to proteins. Thus, while these results serve to validate the recycling concept, they also underscore the need for new, highly efficient bioconjugation reactions that can be carried out on process scale. The development of such methods serves as a major objective in our research, as they will be of key importance to increasing the practicality of these and other bioconjugates for materials applications.

dx.doi.org/10.1021/ja309277v | *J. Am. Chem. Soc.* 2013, 135, 293–300

■ ASSOCIATED CONTENT

Supporting Information

Additional procedures and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

mbfrancis@berkeley.edu

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

These studies were generously supported by the Energy Biosciences Institute at the University of California, Berkeley. K.J.M. was supported by a Graduate Fellowship from the NSF (2011119971) and the U.C. Berkeley Chemical Biology Graduate Program (NIH Training Grant 1 T32 GMO66698). The authors gratefully acknowledge Prof. Douglas Clark for the EGPh-containing plasmid used in this work. Prof. Jean M. J. Fréchet, Prof. Michelle Chang, and Prof. Christopher J. Chang are also acknowledged for instrumentation access.

■ REFERENCES

(1) Yeoman, C. J.; Han, Y.; Dodd, D.; Schroeder, C. M.; Mackie, R. I.; Cann, I. K. O. *Adv. Appl. Microbiol.* **2010**, *70*, 1−55.

(2) Klein-Marcuschamer, D.; Oleskowicz-Popiel, P.; Simmons, B. A.; Blanch, H. W. *Biotechnol. Bioeng.* **2012**, *109*, 1083−1087.

(3) Lynd, L. R.; Laser, M. S.; Bransby, D.; Dale, B. E.; Davison, B.; Hamilton, R.; Himmel, M.; Keller, M.; McMillan, J. D.; Sheehan, J.; Wyman, C. E. *Nat. Biotechnol.* **2008**, *26*, 169−172.

(4) Ogeda, T. L.; Silva, I. B.; Fidale, L. C.; El Seoud, O. A.; Petri, D. F. S. *J. Biotechnol.* **2012**, *157*, 246−252.

(5) Lupoi, J. S.; Smith, E. A. *Biotechnol. Bioeng.* **2011**, *108*, 2835−2843.

(6) Mandali, P.; Dalaly, B. K. *J. ASTM Int.* **2010**, *7*, 1−10.

(7) Andriani, D.; Sunwoo, C.; Ryu, H.-W.; Prasetya, B.; Park, D.-H. *Bioprocess. Biosyst. Eng.* **2012**, *35*, 29−33.

(8) Jordan, J.; Kumar, C. S. S. R.; Theegala, C. *J. Mol. Catal. B-Enzym.* **2011**, *68*, 139−146.

(9) Xu, J.; Huo, S.; Yuan, Z.; Zhang, Y.; Xu, H.; Guo, Y.; Liang, C.; Zhuang, X. *Biocatal. Biotransform.* **2011**, *29*, 71−76.

(10) Taniguchi, M.; Kobayashi, M.; Fujii, M. *Biotechnol. Bioeng.* **1989**, *34*, 1092−1097.

(11) Liang, W.; Cao, X. *Bioresour. Technol.* **2012**, *116*, 140−146.

(12) Zhang, Y.; Xu, J.-L.; Li, D.; Yuan, Z.-H. *Biocatal. Biotransform.* **2010**, *28*, 313−319.

(13) Hoffman, A. S.; Stayton, P. S. *Prog. Polym. Sci.* **2007**, *32*, 922−932.

(14) Broyer, R. M.; Grover, G. N.; Maynard, H. D. *Chem. Commun.* **2011**, *47*, 2212−2226.

(15) Ding, Z.; Chen, G.; Hoffman, A. S. *Bioconjugate Chem.* **1996**, *7*, 121−125.

(16) Shimoboji, T.; Larenas, E.; Fowler, T.; Hoffman, A. S.; Stayton, P. S. *Bioconjugate Chem.* **2003**, *14*, 517−525.

(17) Bontempo, D.; Li, R. C.; Ly, Y.; Brubaker, C. E.; Maynard, H. D. *Chem. Commun.* **2005**, 4702−4704.

(18) Chang, C.-W.; Nguyen, T. H.; Maynard, H. D. *Macromol. Rapid Commun.* **2010**, *31*, 1691−1695.

(19) Kim, H.-W.; Ishikawa, K. *Proteins* **2010**, *78*, 496−500.

(20) Esser-Kahn, A. P.; Francis, M. B. *Angew. Chem., Int. Ed.* **2008**, *47*, 3751−3754.

(21) Esser-Kahn, A. P.; Iavarone, A. T.; Francis, M. B. *J. Am. Chem. Soc.* **2008**, *130*, 15820−15822.

(22) Kim, T.-W.; Chokhawala, H. A.; Nadler, D.; Blanch, H. W.; Clark, D. S. *Biotechnol. Bioeng.* **2010**, *107*, 601−610.

(23) Witus, L. S.; Moore, T.; Thuronyi, B. W.; Esser-Kahn, A. P.; Scheck, R. A.; Iavarone, A. T.; Francis, M. B. *J. Am. Chem. Soc.* **2010**, *132*, 16812−16817.

(24) Schlick, T. L.; Ding, Z.; Kovacs, E. W.; Francis, M. B. *J. Am. Chem. Soc.* **2005**, *127*, 3718−3723.

(25) Christman, K. L.; Broyer, R. M.; Tolstyka, Z. P.; Maynard, H. D. *J. Mater. Chem.* **2007**, *17*, 2021−2027.

(26) Schild, H. G. *Prog. Polym. Sci.* **1992**, *17*, 163−249.

(27) Kim, H. K.; Park, T. G. *Enzyme Microb. Technol.* **1999**, *25*, 31−37.

(28) Starovoytova, L.; Spevacek, J.; Hanykova, L.; Ilavsky, M. *Macromol. Symp.* **2003**, *203*, 239−246.

(29) Ando, S.; Ishida, H.; Kosugi, Y.; Ishikawa, K. *Appl. Environ. Microbiol.* **2002**, *68*, 430−433.

(30) Gilmore, J. M.; Scheck, R. A.; Esser-Kahn, A. P.; Joshi, N. S.; Francis, M. B. *Angew. Chem., Int. Ed.* **2006**, *45*, 5307−5311.

(31) Dixon, H. B. F. *J. Protein Chem.* **1984**, *3*, 99−108.

(32) Andric, P.; Meyer, A. S.; Jensen, P. A.; Dam-Johansen, K. *Biotechnol. Adv.* **2010**, *28*, 308−324.

(33) Griggs, A. J.; Stickel, J. J.; Lischeske, J. J. *Biotechnol. Bioeng.* **2012**, *109*, 676−685.

(34) Eriksson, R.; Borjesson, J.; Tjerneld, F. *Enzyme Microb. Technol.* **2002**, *31*, 353−364.

(35) Sipos, B.; Szilagyi, M.; Sebestyen, Z.; Perazzini, R.; Dienes, D.; Jakab, E.; Crestini, C.; Reczey, K. *C.R. Biologies* **2011**, *334*, 812−823.

(36) Somerville, C.; Youngs, H.; Taylor, C.; Davis, S. C.; Long, S. P. *Science* **2010**, *329*, 790−792.