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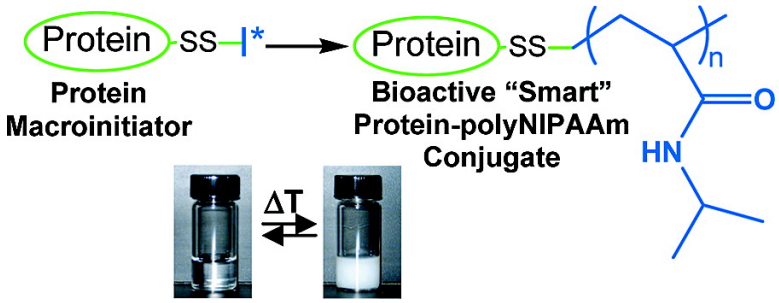

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In Situ Preparation of Protein−"Smart" Polymer Conjugates with Retention of Bioactivity

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Abstract: Protein−polymer conjugates are widely used in biotechnology and medicine, and new methods to prepare the bioconjugates would be advantageous for these applications. In this report, we demonstrate that bioactive "smart" polymer conjugates can be synthesized by polymerizing from defined initiation sites on proteins, thus preparing the polymer conjugates in situ. In particular, free cysteines, Cys-34 of bovine serum albumin (BSA) and Cys-131 of T4 lysozyme V131C, were modified with initiators for atom transfer radical polymerization (ATRP) either through a reversible disulfide linkage or irreversible bond by reaction with pyridyl disulfide- and maleimide-functionalized initiators, respectively. Initiator conjugation was verified by electrospray-ionization mass spectroscopy (ESI-MS), and the location of the modification was confirmed by μLC-MSMS (tandem mass spectrometry) analysis of the trypsin-digested protein macroinitiators. Polymerization of *N*-isopropylacrylamide (NIPAAm) from the protein macroinitiators resulted in thermosensitive BSA−polyNIPAAm and lysozyme−polyNIPAAm in greater than 65% yield. The resultant conjugates were characterized by gel electrophoresis and size exclusion chromatography (SEC) and easily purified by preparative SEC. The identity of polymer isolated from the BSA conjugate was confirmed by ^{1}H NMR, and the polydispersity index was determined by gel permeation chromatography (GPC) to be as low as 1.34. Lytic activities of the lysozyme conjugates were determined by two standard assays and compared to that of the unmodified enzyme prior to polymerization; no statistical differences in bioactivity were observed.

Introduction

In nature, proteins have evolved for highly specialized biological functions and as a result are ideal for various applications in both medicine and biotechnology. Properties for such applications have been improved by the attachment of synthetic polymers to form protein−polymer conjugates.[1,2] For example, covalent modification of proteins with poly(ethylene glycol) (PEG), termed PEGylation, has found its niche in therapeutics, and various PEGylated proteins have improved biodistribution and pharmacokinetics, reduced immunogenicity, prolonged plasma half-life, and increased solubility compared to the non-PEGylated counterparts.[3] Much interest has also been focused on developing protein−polymer conjugates for use as molecular sensors for diagnostic assays, for example, to detect HIV antibodies,[4−7] to study enzyme inactivation,[8] and for switches.[9−12] In this context, several groups have worked on developing "smart" protein−polymer conjugates, employing the temperature responsive poly(*N*-isopropylacrylamide) (polyNIPAAm).[13,14] The thermoresponsive nature of polyNIPAAm manifests in a characteristic lower critical solution temperature (LCST) in water at 32 °C, above which polyNIPAAm undergoes a hydrophobic collapse and phase separates from solution.[14] The polymer confers this temperature sensitivity to the protein to which it is attached. Hoffman, Stayton, and others have demonstrated the usefulness of protein−polyNIPAAm conjugates in enzyme recovery,[15] for triggered release or blocking of substrates to protein active sites,[16,17] and regulation of enzyme activity[18] through thermal precipitation.

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Preparation of polymer bioconjugates is generally achieved by reaction of preformed semitelechelic polymers with specific amino acid residues. Lysine side chains are frequently targeted with amine-reactive end-groups such as activated esters, isocyanates, or through reductive amination.[19−21] However, a protein may contain several internal lysine residues in addition to the N-terminal amine. Therefore, this approach is often nonspecific, and the resultant protein−polymer conjugate is heterogeneous in the number and placement of the polymer chains. Heterogeneity in the structure reflects on the biological properties of the conjugate and often results in decreased protein activity.[22] Hence, creating well-defined adducts is important, and site-specific modification of the protein is a better approach to prepare such biomolecules. Site-specific modification of proteins is also important for directed immobilization onto surfaces and ensures that biorecognition sites are accessible.[6] In addition, for "smart" polymer switches, placement of the polymer chain near the protein or enzyme active site is critical for reversible activity control.[16] Self-assembly of enzymes and proteins modified with hydrophobic chains also requires well-defined conjugates.[23,24]

Various creative methods have been explored to obtain site-specific bioconjugates. Examples include oxime formation by reaction of ketone-modified tyrosine residues[25] or lysine residues[22,26] with aminooxy end-functionalized PEGs. Cofactor reconstitution between an enzyme and polymer[27] has also proven to be an efficient strategy. Polymers with ligands for protein binding sites are used; for example, biotinylated polymers that bind to streptavidin or avidin have been synthesized.[24,28−31] Furthermore, Griffith et al. have made use of affinity interactions by the preparation of protein−polymer conjugates consisting of polymers with side chain Ni^{2+} complexes and a polyhistidine-tagged growth factor.[32]

Cysteine residues are frequently targeted for site-specific conjugation by exploiting thiol chemistry. Generally, proteins contain very few, if any, cysteines that do not participate in disulfide bonds. Therefore, by making available and then targeting free cysteines the number and placement of polymer chains on the protein can be precisely determined. In addition, if a protein lacks free thiols for conjugation, genetic engineering can incorporate cysteine residues in specific positions, for example away from the active site, such that a polymer can be attached without hindering protein activity.[1,33] We have recently demonstrated that atom transfer radical polymerization (ATRP) can be used to prepare pyridyl disulfide semitelechelic poly(2-hydroxyethyl methacrylate) that without any postpolymerization modification conjugates to bovine serum albumin (BSA) via a reversible disulfide bond.[34] Maleimide end-functionalized poly(PEG methacrylate) has also been prepared by ATRP and conjugated to BSA and to glutathione through covalent C−S attachment of the polymer.[35] Other reactive groups which are often employed for thiol conjugation include vinyl sulfone[36] and iodoacetamide.[37]

Each of these approaches to prepare protein−polymer conjugates attaches a preformed polymer to the protein. Most often an excess of polymer is used, which must be removed from the conjugate; this can be difficult to achieve if the polymer and protein are similar in size. In addition, it can be difficult to determine the number and location of the polymer chains in the final conjugate, particularly when multiple attachment sites are possible. Recently, we reported an alternative way to prepare protein−polymer conjugates that circumvents these issues.[38] The approach involves first modifying the protein with initiation sites for polymerization and then polymerizing from the protein macroinitiator to form the bioconjugate in situ. Polymers had previously been grafted from proteins by randomly generating radicals on amino acid side chains,[39−43] although in these examples, the number and sites of polymerization could not be controlled. However, by first modifying the protein, we showed that the polymerization is initiated from specific domains, and the resultant locations of polymer conjugation can be predetermined. Specifically, the protein streptavidin (SAv) was modified with a biotinylated initiator and used for the formation of the conjugate in situ.[38] The initiation sites were defined by placement of the modified biotins, and conjugate formation was efficient. This methodology has the advantage that compared to the traditional approach, purification is simplified: (1) unreacted small molecules such as residual monomer are readily removed by simple dialysis or chromatography; and (2) determination of the number and placement of initiation sites, and therefore resulting polymer, is readily achieved by mass spectrometry techniques. Because the macroinitiator was formed by the interaction of functionalized biotin, the identity of the protein in this example was limited to those that bind the ligand, namely streptavidin, avidin, and recombinant derivatives such as NeutrAvidin.

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To explore the generality of the approach, we modified proteins at free cysteine residues and determined resultant conjugate bioactivity. This amino acid was targeted because well-defined bioconjugates are typically prepared by reacting preformed polymer chains with free cysteines that exist naturally or are placed in mutant forms of proteins. Therefore, polymerizing from proteins modified at cysteine residues represents a general strategy to protein−polymer conjugates and offers the possibility to modify proteins at specific positions. Herein, we report the modification of BSA and a mutant form of lysozyme bearing a free cysteine with either a disulfide- or S−C-linked initiator. Subsequent polymerization of NIPAAm using the macroinitiators formed the "smart" polymer conjugates in situ. Lysozyme is a small enzyme that provides protection from bacteria by breaking down polysaccharide walls;[44] enzymatic activity of the lysozyme−polyNIPAAm was assessed and compared to that of unmodified mutant lysozyme to demonstrate that bioactivity is unaffected by the polymerization process. The results illustrate that the lysozyme survives the polymerization conditions and that bioactivity is unaffected by polymer conjugation.

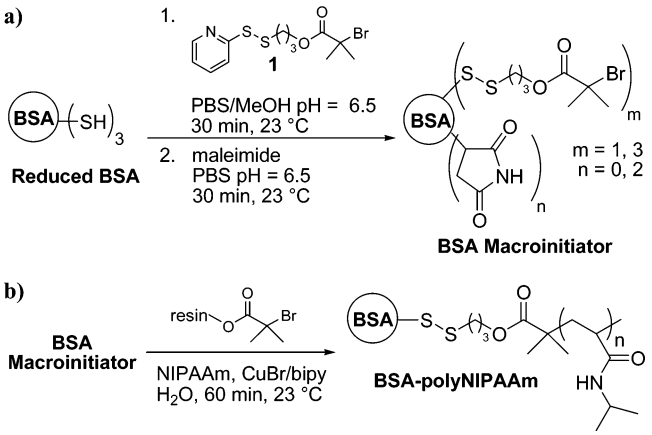
Results and Discussion

Preparation of the Bovine Serum Albumin (BSA) Macroinitiator. The approach was first explored using BSA for several reasons. BSA is readily available in larger quantities than most proteins which provided us flexibility to explore the polymerization conditions and generate enough bioconjugate to cleave and isolate the resultant polyNIPAAm. Therefore, we could confirm the technique and characterize the polymer prior to utilization of the mutant lysozyme. The lysozyme was expressed and isolated as a bioconjugate in small quantities, and it was not possible to isolate and characterize the resulting polymer. BSA also contains a free surface cysteine at amino acid 34.

Although BSA contains a free cysteine, it is known that partial oxidation at Cys-34 results in only ∼50% of the residue being available for conjugation.[45] Therefore, to maximize the number of thiols present for initiator conjugation, BSA was first reduced with tris-(2-carboxyethyl) phosphine hydrochloride (TCEP). Quantification using Ellman's assay[46] confirmed that, after reduction, the average percentage of free thiols increased from approximately 50% to 300%. This indicated that one disulfide bond in the protein cleaved and that three thiols were available per BSA molecule for subsequent conjugation to the thiol-reactive pyridyl disulfide initiator **1**.

Initiator conjugation proceeded by adding a solution of **1** in methanol to the reduced BSA in phosphate-buffered saline (PBS) and stirring under argon for 30 min to form the disulfide-linked conjugate (Scheme 1a). Thiols are excellent chain transfer agents,[47] and unmodified cysteines could be detrimental to the subsequent polymerization reaction. Although this is unlikely to be problematic when small amounts of protein are utilized, to exclude this possibility the resulting BSA−initiator conjugate

Scheme 1 [a,b]



[a] Modification of BSA with the initiator fragment and "capping" of reduced BSA to form the BSA macroinitiator. [b]Polymerization from BSA macroinitiator in the presence of a 2-bromoisobutyrate-functionalized resin.

was reacted with maleimide to "cap" any unmodified thiols (Scheme 1a). Ellman's assay confirmed that free thiols were no longer present, meaning that every cysteine had reacted with **1**, maleimide, or reformed a disulfide bond. Electrospray-ionization mass spectrometry (ESI-MS) was employed to monitor each step of the reaction; the results (see the Supporting Information for spectra and analysis) confirmed that BSA was modified with initiation sites. Intense peaks at masses 66 680 amu and 66 869 amu were observed for the BSA macroinitiator corresponding to BSA modified with one initiator, without and with maleimide conjugation, respectively. The protein with three initiators was also detected (67 157 amu), indicating that BSA with either one or three polymer chains attached was possible after polymerization. Tandem mass spectrometry analysis (μLC-MSMS) of the trypsin digest peptides for the BSA−macroinitiator showed that Cys-34 was modified with the initiator. The monoisotopic masses of the peptide fragment containing Cys-34 derived from the macroinitiator and unmodified BSA were 2674.21 and 2435.25 amu, respectively. The difference (238.96 amu) corresponded exactly to the expected mass of the initiator.

Polymerization from BSA. BSA was modified with a typical initiator for ATRP,[48,49] a 2-bromoisobutyrate group, to start the polymerization from defined sites on the protein.[38] NIPAAm was polymerized from the BSA macroinitiator in water at ambient temperature (Scheme 1b) using the catalyst system copper bromide/2,2′-bipyridine (CuBr/bipy) in the presence of 2-bromoisobutyryl-functionalized resin (the initial [NIPAAm]/[resin]/[CuBr]/[bipy], where [resin] indicates the estimated concentration of initiating sites on the resin, was 100:1:1:2). The "sacrificial" resin-bound initiator increases the total initiator concentration and thus enables polymerization at very low macroinitiator concentrations.[38,50] Because many proteins are available only in small quantities, flexibility in this respect is essential. However, BSA is commercially available in large quantities, and therefore polymerization without the "sacrificial" initiator was also demonstrated (initial [NIPAAm]/[BSA macroinitiator]/[CuBr]/[bipy] ratio of 254:1:1:2). Removal of the polymer-coated resin from the protein−polymer conjugate

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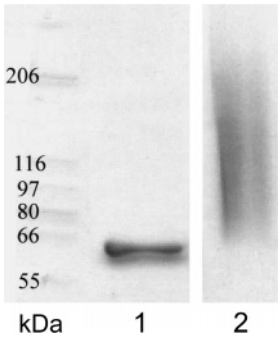

Figure 1. SDS−PAGE of isolated BSA−polyNIPAAm. Lane 1: BSA−polyNIPAAm, reducing conditions with 2% β-mercaptoethanol. Lane 2: BSA−polyNIPAAm, nonreducing conditions.

after the former polymerization was easily achieved by centrifugation, and for both polymerizations, the characteristic thermal precipitation of the polyNIPAAm allowed for immediate detection of conjugate formation. Analysis of the crude reaction mixtures by SEC showed that 65% of the BSA was modified with polyNIPAAm. Polymerization in the absence of the "sacrificial" resin resulted in 44% of BSA−polyNIPAAm conjugate. Conjugate formation was also verified by SDS-PAGE.

Two control experiments were performed on this system. First, NIPAAm was polymerized from the initiator-functionalized resin in the presence of maleimide-capped BSA, and second, unmodified BSA was incubated with NIPAAm monomer and the catalyst to determine if the polymerization could be initiated in the absence of the 2-bromoisobutyrate group. Analysis by SDS−PAGE indicated that in both cases polymer had not formed; the protein after polymerization did not shift to higher molecular weight (see the Supporting Information). These results indicate that the bioconjugate does not form via chain transfer reactions and that the protein must be modified with initiation sites for polymerization to occur.

Characterization of BSA−polyNIPAAm Conjugates and Polymer. Prior to product isolation, GPC studies were carried out to rule out the presence of any free polyNIPAAm in solution. Small aliquots of the reaction mixture after polymerization were lyophilized and then redissolved in DMF. The differential solubility in DMF (BSA is insoluble and polyNIPAAm is soluble) was exploited to selectively extract any possible free polymer. In both cases, polymer was not observed by GPC, indicating that no free polyNIPAAm was formed in solution. Therefore, the observed thermosensitivities were due to polymer covalently attached to BSA.

Isolation of the BSA−polymer conjugates from unmodified BSA and unreacted monomer was accomplished by preparative SEC. This was readily achieved because of the differences in retention times for the species. After purification of the conjugate prepared in the presence of "sacrificial initiator", only a small amount of unmodified BSA was present in the conjugate sample, demonstrating the effectiveness of the purification procedure (see the Supporting Information). After lyophilization, this conjugate was analyzed by SDS−PAGE (Figure 1). Under nonreducing conditions, the conjugate appeared as a broad band higher in molecular weight than BSA. The sample was also heated in the presence of the reducing agent β-mercaptoethanol and then analyzed. Under these conditions, the polymer was cleaved from the protein and only the BSA band was visible in the SDS−PAGE gel. These results indicate that, as expected, the polymer is conjugated to the protein via a reversible disulfide bond.

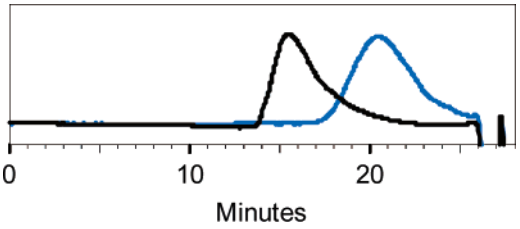

Figure 2. GPC traces of isolated polyNIPAAm polymerized in the presence of resin (blue) and in the absence (black).

The disulfide linkage was exploited to isolate and characterize the polymer formed on the protein. PolyNIPAAm was cleaved from BSA by subjecting the conjugates to a reducing agent, dithiothreitol (DTT). The BSA−polyNIPAAm conjugates were treated with a 1 mg/mL solution of DTT in DMF for 3 days at 60 °C. The reduction was performed in DMF in order to avoid precipitation of the polymer. GPC traces (Figure 2) of the cleaved polymers exhibited low molecular weight tailing which can be attributed to termination reactions early during the polymerization. Nevertheless, the chromatograms were monomodal. Polydispersity indices of the polyNIPAAm polymerized in the presence and absence of resin were 1.34 (number average molecular weight = 11 300) and 1.50 (number average molecular weight = 58 300), respectively. The identity of the polyNIPAAm was confirmed by ^{1}H NMR analysis.

For comparison, polymerization of NIPAAm in water was evaluated in similar conditions as polymerization from the BSA, using a water soluble 2-bromoisobutyryl oligo(ethylene glycol) initiator.[51] In this system a polydispersity of 3.25 was obtained. Although a different catalyst was employed (copper chloride/bipy) this may not explain the differences in molecular weight distribution. We speculate that the lower polydispersity obtained when polymerizing from the protein macroinitiator is due to site isolation of the growing radicals on the BSA, thereby reducing termination events, which ultimately results in the narrower molecular weight distributions for the polymer. In addition, polymers in solution can freely diffuse, and thus the rate of termination should be faster compared to protein-bound polymer; differences in diffusion have been attributed to differences in termination rates for polymers in solution and those grown from nanoparticles.[52]

Taken together, the results clearly demonstrate that proteins modified with initiation sites through disulfide bonds can be used as macroinitiators for polymerization. The resulting conjugates are readily purified, and the polymers formed have narrower molecular weight distributions than those polymerized under similar conditions in solution. No free polymer is detected at the end of the polymerization, and the polymer is released under reducing conditions indicating that the polymer is conjugated to the protein through a disulfide bond. However, for the approach to be viable for many applications, the bioactivity of the resulting conjugate must not be compromised during polymerization. Because bioactivity would be difficult to assess using the model protein BSA, modification of a bioactive enzyme was pursued.

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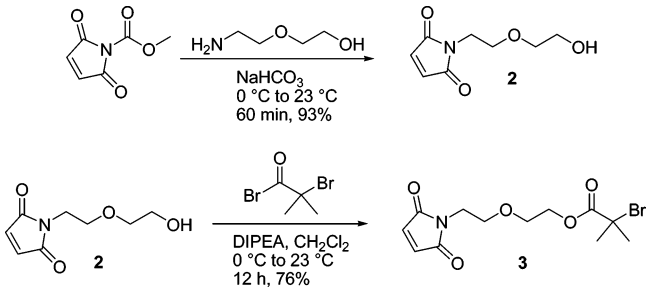
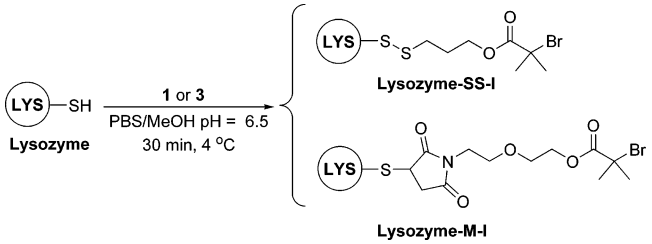
Scheme 2. Synthesis of the Maleimide-Functionalized Initiator



Scheme 3. Conjugation of Initiation Sites to Lysozyme



Modification of Lysozyme Mutant V131C. Natural forms of lysozyme do not contain free thiols; therefore, we chose a genetically engineered "V131C" mutant of T4 lysozyme bearing a single cysteine that conferred one free thiol to the protein for subsequent modification. Employing *E. coli* host BL21(DE3) and the expression vector for V131C, the mutant was expressed and isolated as described by Hubbell and co-workers[53,54] and then purified by cation exchange chromatography and preparative SEC. SDS−PAGE under reducing and nonreducing conditions indicated that the mutant was isolated as a mixture of dimer and monomer. Therefore, initiator conjugation was preceded by reduction using immobilized TCEP disulfide reducing gel.

The single cysteine of lysozyme mutant V131C (hereafter referred to as "lysozyme") was modified via a disulfide bond using the pyridyl disulfide initiator **1** and through an irreversible C−S bond using the maleimide end-functionalized initiator **3**. The maleimide initiator **3** was synthesized in two steps from commercially available *N*-methoxycarbonyl maleimide. Maleimidoethoxyethanol **2** was synthesized according to literature[55] and then esterified with 2-bromoisobutyryl bromide in the presence of *N*,*N*-diisopropylethylamine to form the maleimide end-functionalized initiator **3** in 76% yield (Scheme 2).

Conjugation of the two initiators to lysozyme proceeded by mixing the enzyme in PBS (pH 6.5) with a solution of either **1** or **3** in MeOH at 4 °C for 30 min (Scheme 3). ESI-MS was conducted on the products, and the data (see the Supporting Information) confirmed that in both cases the lysozyme was modified with one initiator. Intense peaks at 18 848 amu (18 846 expected) for the disulfide-initiator-modified lysozyme and at 18 942 amu (18 942 expected) for the maleimide-modified lysozyme were observed. Although it does not rule out the possibility of unmodified protein, none was detected. A trypsin digestion was performed on the lysozyme−disulfide initiator, and the high cross correlation coefficient (2.88) obtained from

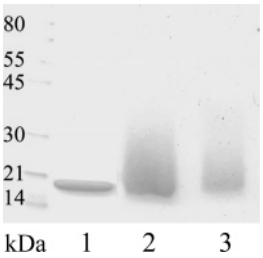


Figure 3. SDS−PAGE of crude lysozyme−polyNIPAAm conjugates. Lane 1: lysozyme. Lane 2: lysozyme−SS−polyNIPAAm. Lane 3: lysozyme−M−polyNIPAAm.

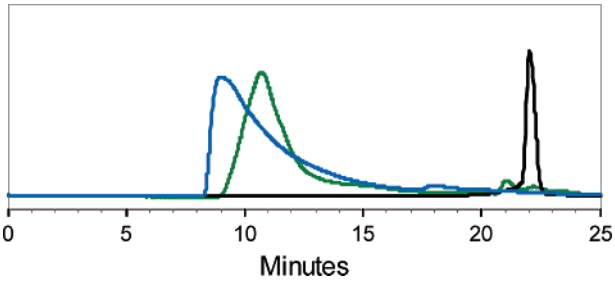


Figure 4. Isolated SEC trace of lysozyme−SS−polyNIPAAm (blue), lysozyme−M−polyNIPAAm (green), and lysozyme (black). Flow rate 0.20 mL/min, 100 mM sodium chloride, 10 mM ammonium acetate in water, pH 6.60; $\lambda = 215$ nm.

the peptide fragment containing the Cys-131 indicated that the lysozyme was indeed modified at the cysteine residue and not through covalent attachment to a different amino acid residue of the protein.

Polymerization from Lysozyme. Polymerization from lysozyme was conducted in water at ambient temperature in the presence of functionalized resin in the same manner as from the BSA macroinitiator. After 90 min, the reaction mixture was exposed to air to stop polymerization, and the resin was removed by centrifugation. Upon warming, the lysozyme−polyNIPAAm prepared from both macroinitiators precipitated, suggesting polymer formation. Turbidity measurements of conjugate samples at 600 nm revealed that the LCSTs of the conjugates were 33−34 °C (see the Supporting Information). Analysis of the crude lysozyme−polyNIPAAm conjugates by SEC showed that polymerization from the disulfide macroinitiator resulted in approximately 75% protein−polymer conjugate formation, and polymerization from the maleimide macroinitiator resulted in 65% protein−polymer conjugate formation. The crude conjugates were also analyzed by SDS−PAGE (Figure 3), and a higher molecular weight band was apparent for both of the lysozyme conjugates compared to that of the unmodified lysozyme. Purification of the conjugates was achieved by preparative SEC, and the traces of the isolated conjugates were significantly shifted to higher molecular weight compared to that of unmodified lysozyme (Figure 4).

Activity of Resultant Lysozyme−polyNIPAAm Conjugates. The activities of the conjugates were evaluated using standard assays and compared to that of the unmodified lysozyme. The first assay measured the lytic ability of the lysozyme with respect to the lyophilized substrate *Micrococcus lysodeikticus.*[56] Lysozyme, lysozyme initiators, and isolated lysozyme conjugates were prepared with equal protein concen-

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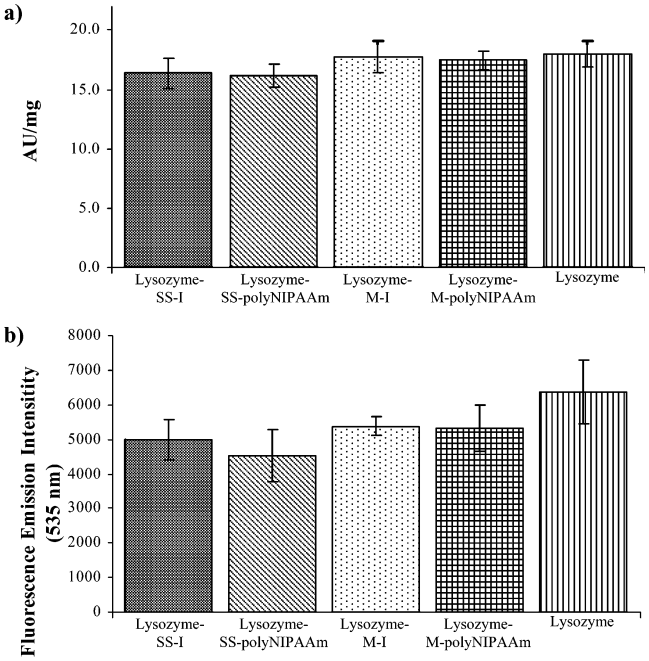

Figure 5. Activity assays of lysozyme and lysozyme conjugates. (a) UV−vis assay of lysozyme activity: average decrease in absorbance over 2 min of a solution of *Micrococcus lysodeikticus* measured at 450 nm. The change in absorbance is due to cell wall lysis from lysozyme activity on the substrate. 1 AU = 0.001 decrease in absorbance/min. Error bars represent the standard deviations. (b) Fluorescence assay of lysozyme activity: fluorescence results from released fluorescein from labeled *Micrococcus lysodeikticus* due to lysozyme activity on the substrate (excitation 485 nm/emission 535 nm). Error bars represent the standard deviations.

trations, and then 100 μL of the enzyme solution was mixed with 600 μL of *M. lysodeikticus* solution. Upon cell wall lysis, the solution becomes less turbid, and this decrease in absorbance was monitored for 2 min at 450 nm. Activity was expressed in activity units (AU). One AU is defined as a change in absorbance of 0.001 per min. These experiments were performed 4 times for each sample, and groups were compared (Figure 5a) using one-way analysis of variance (ANOVA). No statistical difference in activities was observed ($P > 0.10$).

The second activity assay performed on the lysozyme conjugates measured the lytic activity toward fluorescein-labeled *Micrococcus lysodeikticus.* Following the manufacture's protocol, in a 384-well plate, lysozyme samples of equivalent protein concentrations were incubated with the labeled substrate for 30 min at 37 °C. Fluorescence emission at 535 nm (excitation at 485 nm) was measured, and the average intensities were compared (Figure 5b, experiments in triplicate). One-way ANOVA analysis indicated no statistical difference between the samples ($P > 0.07$). Therefore, conjugation of the lysozyme with an initiator for polymerization, or with an eventual polymer, did not result in a decrease of enzyme activity; bioactivity was fully retained.

These results verify with two different systems, BSA and lysozyme, that protein−polymer conjugates can be prepared by polymerization from the modified proteins and readily isolated. The initiation site can be linked through a disulfide bond or through an irreversible C−S bond. Furthermore, the preparative conditions are not detrimental to bioactivity; it was demonstrated that the enzymatic activity of lysozyme is completely retained after polymerization. Cysteines are a common handle to produce well-defined conjugates, and this work demonstrates for the first time that proteins can be modified through cysteine residues and utilized as macroinitiators for polymerization. These results provide evidence that this strategy, which has many advantages over traditional synthetic methods, is a promising, flexible approach to preparing bioactive protein−polymer conjugates.

Conclusion

Protein−polymer conjugates are important materials for various applications. In particular, protein−polyNIPAAm conjugates are promising for the development of sensors and enzyme assays. We have illustrated a method for the preparation of such materials by conjugating initiator moieties to free thiol residues on proteins and polymerizing from these protein−initiator complexes. Specifically, BSA−polyNIPAAm and lysozyme−polyNIPAAm were formed and readily purified by preparative SEC. We demonstrated that the lysozyme−polyNIPAAm conjugates exhibited equivalent activities to that of the unmodified enzyme, meaning that the polymerization process is not detrimental to the enzyme. Polymerization from initiator-conjugated proteins represents an advantageous alternative to the attachment of preformed polymer chains to proteins; compared to the latter method, the conjugates may be more easily purified and characterized. Our results indicate that this polymerization strategy in aqueous solvents to site-specifically modify proteins with polymer chains is a promising approach to bioactive protein−polymer conjugates.

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Supporting Information Available: Experimental section, mass spectrometry data, SDS−PAGE and SEC of crude BSA−polyNIPAAm conjugates, SEC of isolated BSA−polyNIPAAm, ^{1}H NMR of isolated polyNIPAAm, SDS−PAGE and GPC traces of control experiments, LCST experiments of lysozyme−polyNIPAAm, complete refs 22, 26, and 50. This material is available free of charge via the Internet at http://pubs.acs.org.

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