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Well-Defined Protein−Polymer Conjugates via *in Situ* RAFT Polymerization

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Abstract: Biotechnology, biomedicine, and nanotechnology applications would benefit from methods generating well-defined, monodisperse protein−polymer conjugates, avoiding time-consuming and difficult purification steps. Herein, we report the *in situ* synthesis of protein−polymer conjugates via reversible addition−fragmentation chain transfer polymerization (RAFT) as an efficient method to generate well-defined, homogeneous protein−polymer conjugates in one step, eliminating major postpolymerization purification steps. A water soluble RAFT agent was conjugated to a model protein, bovine serum albumin (BSA), via its free thiol group at Cys-34 residue. The conjugation of the RAFT agent to BSA was confirmed by UV−visible spectroscopy, matrix-assisted laser desorption ionization - time of flight (MALDI-TOF), and ^{1}H NMR. BSA−macroRAFT agent was then used to control the polymerization of two different water soluble monomers, *N*-isopropylacrylamide (NIPAAm) and hydroxyethyl acrylate (HEA), in aqueous medium at 25 °C. The growth of the polymer chains from BSA−macroRAFT agent was characterized by size exclusion chromatography (SEC), ^{1}H NMR, MALDI-TOF, and polyacrylamide gel electrophoresis (PAGE) analyses. The controlled character of the RAFT polymerizations was confirmed by the linear evolution of molecular weight with monomer conversion. The SEC analyses showed no detectable free, nonconjugated polymer formation during the *in situ* polymerization. The efficiency of BSA−macroRAFT agent to generate BSA−polymer conjugates was found to be ca. 1 by deconvolution of the SEC traces of the polymerization mixtures. The structural integrity and the conformation-related esterase activity of BSA were found to be unaffected by the polymerization conditions and the conjugation of the polymer chain. BSA−poly(NIPAAm) conjugates showed hybrid temperature-dependent phase separation and aggregation behavior. The lower critical solution temperature values of the conjugates were found to increase with the decrease in molecular weight of poly(NIPAAm) block conjugated to BSA.

Introduction

Increasing utility of polymer conjugates of proteins in medicine,[1−9] biotechnology,[10−15] and nanotechnology[1,15−20] has created the need for generating homogeneous and defined conjugate masses manifesting uniformity in biohybrid properties such as biological activity. Exploration of new preparation methods minimizing the inherent heterogeneity of protein−polymer conjugates has recently revealed the potential of the controlled radical polymerization (CRP) techniques to generate protein−polymer conjugates with defined properties. The nitroxide-mediated polymerization,[21−24] atom transfer radical

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polymerization (ATRP),[25−33] and reversible addition−fragmentation chain transfer (RAFT)[34−45] technique have been used for the synthesis of well-defined end-group-functionalized polymers that can be directly conjugated to proteins without the need for postpolymerization end-group modifications. While the use of narrow disperse polymers bearing defined functional end groups are useful to reduce the number of steps, the yield of conjugation reactions between polymer and protein still limits control over the number of polymers conjugated per protein and thus leads to the generation of heterogeneous mixtures composed of the free polymer and protein, and the conjugates varying in composition. To remove the nonconjugated polymer and protein from the conjugates, tedious purification steps are required, which lowers the overall feasibility of the approach for large-scale applications.

A major improvement toward the generation of well-defined polymer-protein conjugates while avoiding extensive purification steps, was recently introduced by Maynard's group[14,46,47] and followed by others.[48,49] Proteins were first modified with ATRP initiator(s) at a defined site, e.g., biotin-binding site of streptavidin,[46] cysteine residues of bovine serum albumin[47,49] and mutant lysoszyme,[47,49] and lysine residues of chymotrypsin.[48] Polymerizations were then performed from the ATRP initiating sites of proteins in the presence or absence of a sacrificial initiator to form protein−polymer conjugates *in situ*.

While these pioneering studies proved that the ATRP technique provides an attractive route to generate well-defined protein−polymer conjugates in one-step and hence provides the opportunity to evade all postpolymerization conjugation strategies and simplify the purification of the final conjugates, they did not provide a detailed investigation of the controlled character of ATRP from protein-macroinitiators (i.e., kinetic investigation of ATRP). Additionally, although there are recent discoveries in the field,[50−54] the variety of the monomers that can be polymerized via ATRP is still relatively limited, which limits the generality of this elegant approach. It has been shown that the ATRP metal catalysts can complex with polar groups[55] such as amide and carboxyl acids. As proteins are molecules composed of numerous polar groups, the use of metal catalyst might be of concern for the applications involving proteins. Nevertheless, metal catalysts could be removed with due attention and care during the purification steps. Some recent discoveries in the field[56−61] might also eliminate or minimize the concerns regarding the removal of metal catalysts.

The potential to control the polymerization of a wider range of monomers in varying solvents including water without using any metal catalyst are apparent advantages of the RAFT polymerization.[39,40,62] The RAFT technique is expected to allow the synthesis of well-defined protein−polymer conjugates in easily detectable quantities using only RAFT agent-modified proteins and common free radical initiators. Depending on the efficiency of the RAFT agent and the type of monomer, the typical monomer:RAFT ratios used in the RAFT-mediated polymerizations[38−40,62−69] are higher than the monomer:initiator ratios used in ATRP-controlled polymerizations.[26,54,61,70]

In a recent communication,[45] we reported the *in situ* formation of bovine serum albumin (BSA)-poly(PEG-acrylate) conjugates via gamma-radiation-initiated RAFT polymerization using a

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BSA macroRAFT agent in the presence and absence of additional free RAFT agent. In our effort to explore the generality of the RAFT technique for *in situ* generation of well-defined protein−polymer conjugates, we report here the *in situ* generation of well-defined BSA−poly(*N*-isopropylacrylamide) (BSA−poly(NIPAAm)) and BSA−poly(hydroxyethyl acrylate) conjugates via aqueous RAFT polymerization performed with a room temperature azo-initiator and a new BSA−macroRAFT agent (without using free (nonprotein) RAFT agent). Our new work improves the general applicability of the RAFT technique in generation of protein−polymer conjugates by presenting a synthetic method completely protein-friendly as well as providing the high retention of bioactivity of *in situ* generated conjugates. In our previous work, a gamma-radiation source (^{60}-cobalt source) was used to initiate polymerizations. A gamma-ray source may not be commonly available and more importantly gamma rays may have detrimental effects on biomolecular structures.[71] Within this study, a room temperature azo-initiator was used as a replacement of gamma-irradiation. Moreover, all the previous reports for ATRP[46−49] as well as our previous report for RAFT[45] presented synthetic methods utilizing mixtures of aqueous and organic solvents as water-insoluble reagents (RAFT agent, ATRP initiator, etc.) were used during the preparation of protein−polymer conjugates. In this new study, a water-soluble RAFT agent was synthesized and utilized for *in situ* preparation of BSA−polymer conjugates in completely aqueous solutions. Water-based chemistry and the elimination of the gamma ray source make the overall method protein-friendly and generally applicable. Another important point which was not provided by our previous study is the investigation of the bioactivity of the conjugates. Our results presented in this paper illustrate the high retention of protein bioactivity after *in situ* conjugate formation and also the high efficiency of the conjugate formation without any detectable free polymer generation. The high retention of bioactivity and the absence of detectable free polymer traces in *in situ*-generated conjugate samples (which were not achieved in our previous study) are important for the feasibility of the RAFT technique to generate well-defined protein−polymer conjugates without the need for postpolymerization purification steps.

Results and Discussion

Preparation of BSA−macroRAFT Agent. As proteins can be easily denaturated in the presence of organic solvents and at elevated temperatures, the RAFT agent to be conjugated to the protein must be soluble in water and also bear a functional group, allowing its conjugation to protein to be performed under mild conditions. Moreover, the RAFT agent should not interact with the protein structure via secondary interactions, e.g., electrostatic and hydrophobic interactions, which may result in the formation of aggregates or multiconjugations per protein. We designed a new RAFT agent fulfilling these conditions. The new RAFT agent is a trithiocarbonate bearing a pyridyl disulfide group and a poly(ethylene glycol) (PEG, $n = 16$, polydispersity index 1.05) segment on its Z- and R-fragments, respectively (Supporting Information, Figures S1−S3). The pyridyl disulfide group provides the functionality to attach the RAFT agent covalently to the free thiol of protein's cysteine residues via disulfide bonds.[30,72] The PEG segment gives the water solubility to the RAFT agent and hence allows the attachment of the RAFT agent to the protein to be performed in aqueous solutions. The length of the PEG segment was chosen to be the shortest possible to give the water solubility to the whole structure and to also avoid disturbing the reactivity of the RAFT agent. The structure and the purity of the RAFT agent were characterized by ^{1}H NMR and electrospray-ionization mass spectroscopy (ESI-MS) (Supporting Information, Figures S4 and S5). In our preliminary experiments, the RAFT agent was found to be efficient in controlling the polymerization of varying monomers in water at ambient temperatures with an efficiency of ca. 0.9 (data not shown).

Bovine serum albumin (BSA) was selected as a model protein as it is readily available and contains a cysteine residue that is not completely oxidized, i.e., Cys-34.[73] Ellman's assay[74−76] performed to determine the free thiol groups in a standard BSA solution revealed that ca. 47 mol % of BSA contains nonoxidized cysteine residue and is available for the attachment of the RAFT agent. As opposed to multisite attachment strategy previously reported by Maynard's group,[47] the RAFT agent was attached to the available free cysteine residue of BSA without attempting to increase the number of free thiols by reduction of the oxidized cysteine bridges. This conjugation strategy, one RAFT agent per BSA, was previously shown to be effective in *in situ* preparation of polymer conjugates of BSA.[49] It is worth to note that the coupling of the RAFT agent to the BSA that contains a mixture of oxidized and nonoxidized cysteine residues would result in a mixture composed of the BSA−macroRAFT agent and the nonmodified BSA even if all the free thiol residues of BSA are conjugated with a RAFT agent. The use of BSA without reducing the oxidized cysteine residues is illustrative in terms of showing the applicability of the RAFT technique to prepare site-specific protein−polymer conjugates *in situ.* It would be possible to generate the conjugates that are free of nonconjugated protein residues, by employing BSA after reducing the oxidized cysteines[47] or any other protein that contains only nonoxidized thiol groups as long as the yield of the coupling reaction between the RAFT agent and the protein is high. The coupling reaction between the pyridyl disulfide-modified RAFT agent and the free thiols of BSA is discussed in the paragraphs below.

The attachment of the RAFT agent to BSA was performed in the presence of large excess of the RAFT agent (20 equiv) in a phosphate buffer solution at pH 6.0 for 14 h at room temperature. After the reaction, the excess of the RAFT agent was removed via molecular size-dependent separation using centrifuge filters with a molecular weight cutoff of 50000 Da (BSA and RAFT agent, ca. 66400 and 1200 g/mol, respectively). UV−vis spectrophotometer analysis of the filtrate solution of the reaction mixture showed the presence of 2-pyridinethione (characteristic UV-absorption at 350 nm),[72,77] the byproduct forming upon the reaction between the pyridyldisulfide group

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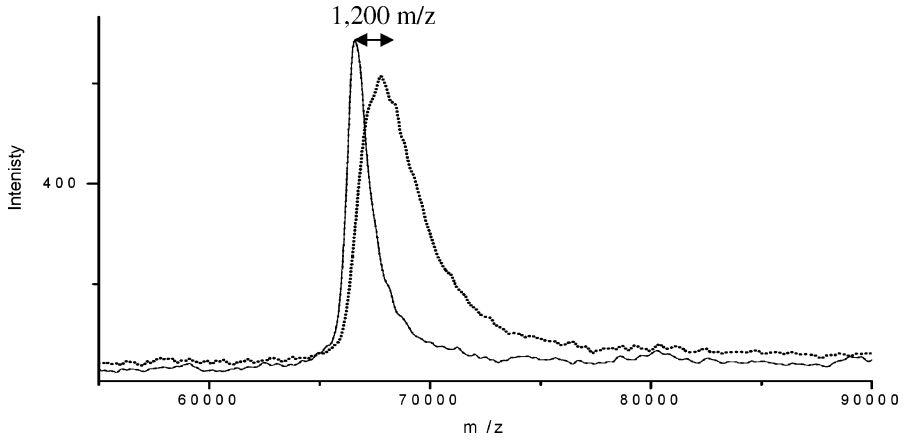


Figure 1. MALDI-TOF chromatograms of BSA before (MW = 66400 Da) and after (MW = 67600 Da) conjugation with the RAFT agent. M_n and PDI of the RAFT agent = 1200 g/mol and 1.06, respectively.

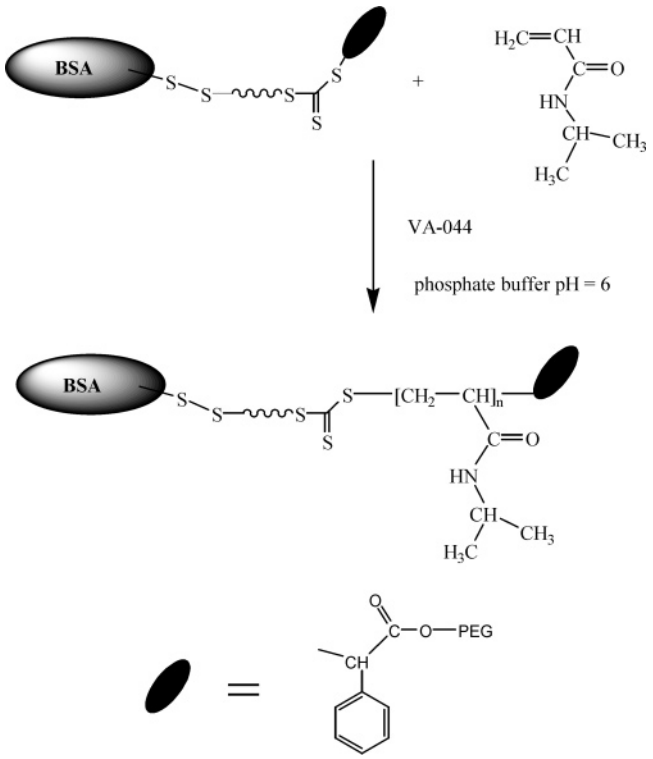
of the RAFT agent and the free thiol of the protein (Supporting Information, Figure S6).

The reaction mixture was washed with buffer solution repetitively using centrifuge filters. As the RAFT agent presents a typical absorption band at ca. 300 nm (Supporting Information, Figure S6), it is easy to analyze the presence of the RAFT agent in the filtrate solutions obtained after each washing step. The absence of the characteristic band in the filtrate solutions after several washing steps showed that the excess RAFT agent was completely removed from the BSA solution. The SEC analysis of purified BSA−macroRAFT agent solution showed one trace with a retention time slightly shorter than BSA's retention time while no trace for the free (nonconjugated) RAFT agent was detectable (Supporting Information, Figure S4). This result further confirmed the complete removal of the excess, nonconjugated RAFT agent from the mixture of BSA−macroRAFT agent and the nonmodified BSA. ^{1}H NMR analysis of the purified sample revealed the characteristic signals of oligoethylene glycol units of the RAFT agent (centered at 3.6 ppm) along with the protein signals (Supporting Information, Figure S8).

Ellman's assay showed almost complete disappearance of the thiol groups of BSA after the reaction with the RAFT agent (thiol concentration before and after the RAFT agent attachment was 33.8 μM and 0.6 μM, respectively, Supporting Information, Figure S9). This result indicated a coupling yield of ca. 96 mol % for the reaction between the free thiol groups of BSA and the RAFT agent (i.e., 96 mol % of the free thiols of BSA was conjugated with a RAFT agent). In other words, ca. 45 mol % of total BSA (of which 47 mol % contains one free thiol available for the attachment of the RAFT agent) was converted to a BSA−macroRAFT agent, leaving the remainder of the BSA unmodified.

The mass of BSA before and after the attachment with the RAFT agent was found to be 66400 and 67600 g/mol, respectively (Figure 1) by MALDI-TOF. The shift in mass corresponded to the addition of one RAFT agent per protein. The broadness of the peak observed with BSA after the attachment of the RAFT agent can be explained by the addition of non-isomolecular PEG-based RAFT agent (polydispersity index, PDI = 1.06) to BSA, widening the molecular weight distribution. It is also important to note that BSA not modified with a RAFT agent due to the oxidized cysteine residues constitutes ca. 55 mol % of total BSA sample.

Scheme 1. *In Situ* Synthesis of BSA−Poly(NIPAAm) Conjugate by RAFT Polymerization Using a BSA−macroRAFT Agent



***In Situ* Formation of BSA−Polymer Conjugates via RAFT Polymerization.** *N*-Isopropylacrylamide (NIPAAm) was polymerized in a phosphate buffer solution at 25 °C using BSA−macroRAFT agent and a free radical initiator, i.e., VA044 (Scheme 1). The pH of the solution was maintained at 6.0 to avoid the possible hydrolysis of the RAFT agent[64] as well as the denaturation of the protein. The ratio of the concentration of monomer to BSA−macroRAFT agent, $[M]_0/[CTA]_0$ was 1800/1. A relatively high ratio of initiator to BSA−macroRAFT agent concentration (4.7/1.0) was used because of the relatively long half-life of the initiator[78] at 25 °C (∼130 h). In typical RAFT polymerizations performed with an usual free radical initiator, e.g., 2,2′-azobisisobutyronitrile, with a half-life at 70 °C of 5 h,[79,80] the ratio of $[initiator]_0/[CTA]_0$ is generally 1.0/

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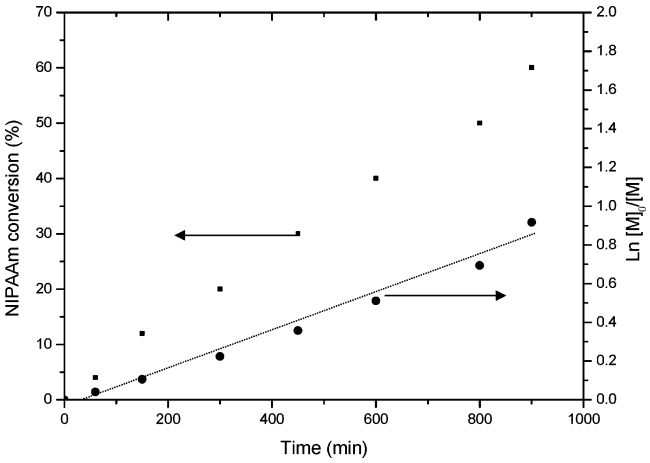

Figure 2. Evolution of NIPAAm conversion and Ln $[M]_0/[M]$ versus time (min) during polymerization performed in the presence of BSA−macroRAFT agent. $[NIPAAm]_0$:$[BSA-macroRAFT]_0$:$[initiator]_0 = 1800.0:1.0:4.7$ in phosphate buffer solution at pH = 6.0 at 25 °C.

5.0.[39,63,66,67,69,81] A similar ratio could be reached in our polymerization system with a starting ratio of ca. 4.7/1.0.

The polymerization mixtures collected at predetermined time intervals were analyzed before purification by aqueous SEC and ^{1}H NMR to determine the evolution of the molecular weight and the monomer conversion, respectively, with time. Figure 2 depicts the evolution of the monomer conversion and Ln $[M]_0/[M]$ versus time. Monomer conversions were calculated by comparing the vinyl resonance ($\delta \sim 5.40$ and 6.30 ppm) of the monomer and the methylene proton resonance ($\delta \sim 3.60$ and 3.85 ppm) of both the monomer and the formed poly(NIPAAm) (Supporting Information, Figure S10). From Figure 2, an inhibition period of 40 min was observed, which can be associated with the slow fragmentation of the intermediate BSA−RAFT agent conjugate[40] or the traces of oxygen. After this first period, the linear evolution of Ln $[M]_0/[M]$ versus time indicated that the system was in stationary state, i.e., radical concentration was constant during polymerization.

The SEC analysis of the polymerization mixtures using refractive index (RI) and UV detectors (at 280 nm) showed the formation of macromolecules having a hydrodynamic volume larger than that of BSA (Figure S11(A), Supporting Information). The RI and UV traces were in good agreement (Figure S11(B), Supporting Information). Since poly(NIPAAm) has negligible absorbance at 280 nm (Supporting Information, Figure S12), the strong UV signals observed with the sample elution (detected by the RI signals) suggested that the traces eluted at lower retention times contained the protein conjugates of poly(NIPAAm). The higher retention time tails of the traces might be attributed to the presence of free BSA not conjugated with a RAFT agent. It was possible to evaluate the proportion of BSA conjugated with polymer (i.e., reacted BSA−macroRAFT agent) to the free BSA (not conjugated with a polymer chain, i.e., nonreacted BSA−macroRAFT agent and nonmodified BSA) in the polymerization mixtures by deconvolution of the UV traces obtained by the SEC analysis (Supporting Information, section 3.4, Figure S13). The comparison of the area of the deconvoluted free BSA peak (retention time: 17.5 min) before and after the polymerization revealed that the proportion of free BSA after the polymerization to the total BSA presented before the polymerization was ca. 54% ± 5. This showed that ca. 46%± 5 of total BSA (nonmodified BSA plus BSA−macroRAFT agent) was converted to a poly(NIPAAm) conjugate (or a structure with a larger hydrodynamic volume) after the polymerizations. Similar results were obtained for the polymerization of hydroxyethyl acrylate (HEA) in the presence of BSA−macroRAFT agent (Supporting Information, Table S1). These deconvolution results were in good agreement with the nonmodified BSA/BSA−macroRAFT ratio used in the polymerization mixtures, determined by Ellman's assay (Supporting Information, Section 3.2.4). Since only 45% of total BSA was BSA−macroRAFT agent (i.e., 55% of BSA could not be modified with a RAFT agent), the results indicated a BSA−macroRAFT agent efficiency of ca. 1.

To investigate whether the aqueous SEC traces of polymerization mixtures might be partially due to the free polymer formed (not conjugated to BSA), a part of the crude polymerization mixtures collected at different monomer conversions were freeze-dried, redissolved in dimethylacetamide (DMAc) for 14 h (at 20 °C), filtered and then analyzed by DMAc SEC. Any free polymer that was not conjugated to the protein should be soluble in DMAc while the free protein along with the protein−polymer conjugates should precipitate and be removed by filtration. Indeed, when the samples were dissolved in DMAc, the precipitate formation was clearly observed. The SEC analysis of the samples, even at high concentrations (10 mg/mL) showed no detectable traces (data not shown), suggesting that the free protein and/or the polymer conjugates of the protein were removed from the solution and more importantly, there were no free polymer present in the solution. This result suggested that the growth of the polymer chains were well-controlled by the BSA−macroRAFT agent during the polymerization. An exception was observed with the DMAc SEC chromatogram of the polymerization mixture having the highest monomer conversion (60%). A high molecular weight trace ($M_n \sim 200000$ g/mol) was observed (data not shown), which was attributed to the BSA conjugate of a high molecular weight polymer chain. A high molecular weight poly(NIPAAm) chain that is highly soluble in DMAc could easily prevent the protein conjugated to the chain from precipitating out.[2,82]

To verify that the *in situ* generated polymer chains were conjugated to BSA via reducible bonds, i.e., via the Z-segment of the RAFT agent attached to BSA, and also to characterize the controlled nature of the polymer growth during the *in situ* polymerization, the crude polymerization mixtures obtained at varying monomer conversions were reacted with a mild, disulfide reducing agent, tris(2-carboxyethyl)phosphine (TCEP). The samples were then freeze-dried and redissolved in DMAc for 14 h. Following filtration to remove the precipitates, the SEC analysis in DMAc revealed the presence of polymer traces (Figure 3) shifting to lower retention times with increasing monomer conversions. This was attributed to the cleavage of *in situ* formed polymer chains from BSA and their subsequent dissolution in DMAc. The appearance of the polymer traces upon reaction with TCEP (no trace detected by DMAc SEC before the reaction with TCEP), proved that the polymer formed during the *in situ* polymerization was covalently

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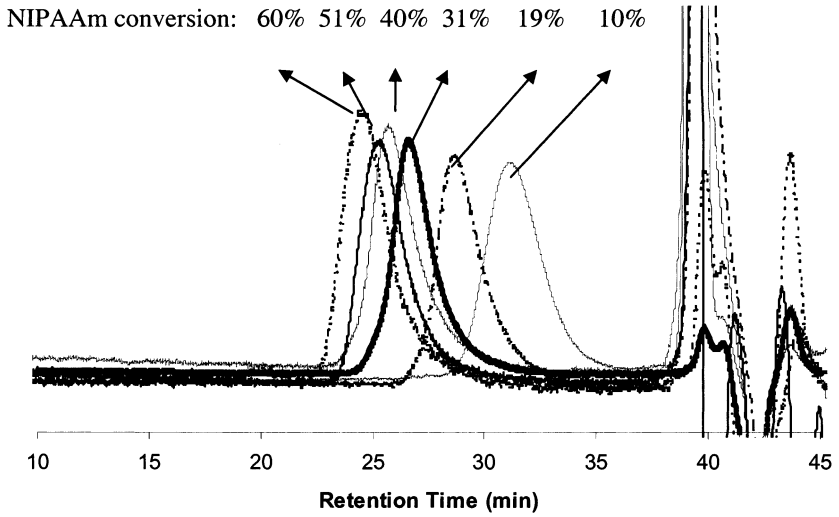

Figure 3. DMAc SEC traces of polymerization mixtures after the reaction with TCEP.

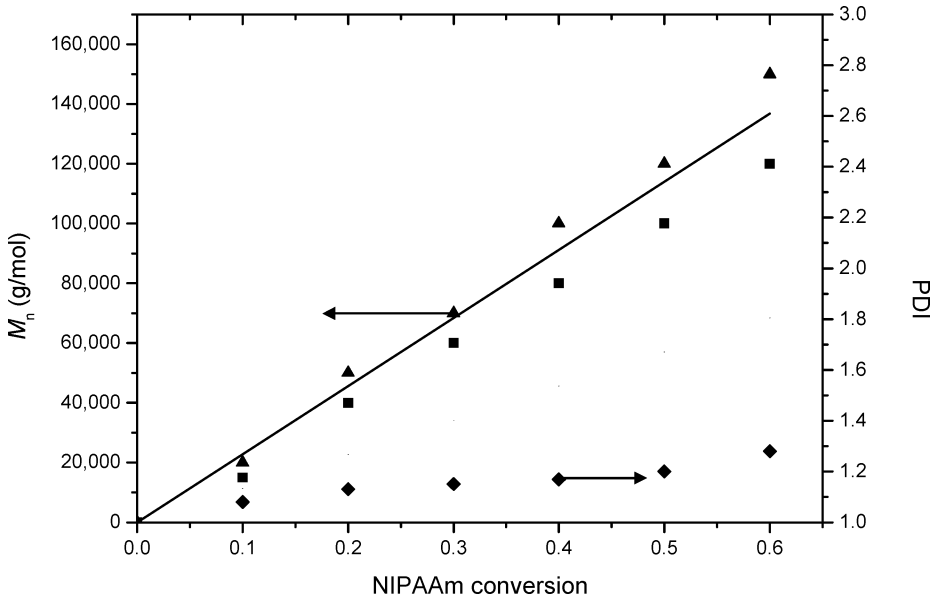

Figure 4. Evolution of the number average molecular weight (M_n) and polydispersity (PDI) values of poly(NIPAAm) cleaved from BSA with monomer conversion (%). Symbol legends: M_n by DMAc SEC (▲) and ^{1}H NMR (■), theoretical M_n values (—) calculated by = $([M]_0/([BSA-macroRAFT\ agent]_0 \times \alpha_M)) \times MW_{NIPAAm}$. $[M]_0$, $[BSA-macroRAFT\ agent]_0$, α_M and MW_{NIPAAm} correspond to the monomer and BSA−macroRAFT agent concentrations, the monomer conversion, and the molecular weight of NIPAAm, respectively.

attached to BSA and the formation of the free polymer (not conjugated to the protein) during the polymerization was none or negligible.

The increase in the molecular weight of the *in situ* grown polymer chains with increasing monomer conversion could be clearly observed from the DMAc SEC analysis of the samples treated with TCEP (Figure 4). The polydispersity index (PDI) of the polymers was less than 1.2 for lower monomer conversions, i.e., < 50%, M_n < 100000 g/mol, while for higher conversion and molecular weight a slight increase in PDI values was observed (PDI < 1.3). The increase in the PDI values with increasing NIPAAm conversion might be due to the steric hindrance effect of the growing polymer chains.[83,84] It would be expected that the RAFT end group of growing BSA−polymer conjugates becomes potentially less accessible, which might decrease the control over the polymerization with increasing monomer conversions.

^{1}H NMR analysis of purified BSA−polymer conjugates (Supporting Information, Figure S14) allowed the determination of the molecular weight of the polymer chains by using the PEG signal of the RAFT-end group at 3.60 ppm as reference signal, and the signals of poly(NIPAAm) at 3.8, 2.00, and 1.00−1.50 ppm that correspond to C*H*$(CH_3)_2$, CH_2, C*H* (backbone chain), and CH_3 of isopropyl group, respectively. It is important to note that the protein signals do not appear on the spectrum under the conditions used for NMR analysis. The molecular weight results obtained by ^{1}H NMR analysis were in good agreement with the values determined by DMAc SEC as well as the theoretical values (Figure 4). The good agreement between the SEC measurements and the theoretical molecular weights in Figure 4 can be considered fortuitous, as PS standards were used for the calibration of SEC measurements.

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The MALDI-TOF spectra of BSA−poly(NIPAAm) conjugates showed the *m*/*z* signals corresponding to the free BSA that was not conjugated with any RAFT agent, along with another signal of 80100 and 102900 Da for the conjugates obtained at 10 and 20% conversions, respectively. The samples with higher conversions did not give clear spectra probably due to the high molecular weight of the conjugates which exceeded the detection limit of the spectrometer. The true molecular weight of the polymers conjugated to BSA was calculated from the difference between the measured values and the molecular weight of BSA (66400 Da) and found to be 13700 and 36500 Da for 10 and 20% monomer conversions, respectively. The molecular weight values obtained from MALDI-TOF measurements were in good agreement with NMR and SEC values.

In a control experiment conducted to verify that the BSA−poly(NIPAAm) conjugates were not formed by a conventional chain transfer polymerization, NIPAAm was polymerized in the presence and absence of BSA (not modified with a RAFT agent) without using a RAFT agent. The DMAc SEC analysis showed a polymer trace with a number average molecular weight of 430000 g/mol (PDI = 3.2) and 450000 g/mol (PDI = 3.3) for the polymerizations with and without BSA, respectively (Supporting Information, Figure S17). The molecules bearing free thiols have been widely used in chain transfer reactions in free radical polymerizations.[85−87] However, similar molecular weight and PDI values of the polymers obtained from polymerizations with or without BSA revealed that the polymer chains formed in the presence of BSA does not anchor to BSA. This suggests that the free thiol on BSA is not reactive toward the chain transfer reactions. These control experiments further confirmed that the conjugates generated during the polymerization of NIPAAm in the presence of BSA−macroRAFT agent were generated via the *in situ* RAFT polymerization.

The RAFT polymerization of hydroxyethyl acrylate (HEA) was also performed in the presence of BSA−macroRAFT agent to confirm the validity of this method to form the well-defined protein conjugates of different polymers (Supporting Information, Figure S15 and S16). The linear evolution of M_n with the monomer conversion suggested that the BSA−poly(HEA) conjugates could be obtained via *in situ* RAFT polymerization using BSA−macroRAFT agent. The relatively high PDI values might be due to the back-biting/transfer reactions of the acrylates.[88,89] Accordingly, gelation of the polymerization mixtures observed at monomer conversions higher than 70% was attributed to the side reactions.

The *in situ* generated BSA−poly(NIPAAm) and BSA−poly(HEA) conjugates with varying molecular weights were analyzed by polyacrylamide gel electrophoresis (PAGE) (Figure 5) for a direct visual confirmation. The conjugates appeared on the gel as higher molecular weight smears while control BSA sample appeared as two distinct bands corresponding to molecular weights of ca. 60000 and 130000 Da. With the polymer conjugate samples, the bands for BSA not modified with a RAFT agent (55 mol % of total BSA) was also observed. With increasing polymer weight (which also reflects increasing monomer conversion), the conjugate smear was found to appear more intense and also run on the gel slower (Figure 5). After the incubation of BSA−poly(NIPAAm) conjugates with TCEP, the smear corresponding to the high molecular weight molecules completely disappeared revealing the bands corresponding to free BSA. This result further confirmed that the polymer was conjugated to BSA via reducible bonds. When a physical mixture of BSA and poly(NIPAAm) (M_n: 140000 g/mol, PDI: 1.75) was run on the gel (Supporting Information, Figure S18), two distinct bands exactly at the same molecular weight level with BSA bands appeared while no smear was observed, indicating that BSA and poly(NIPAAm) does not form conjugates via physical interactions and the *in situ* RAFT generated BSA−poly(NIPAAm) samples were not the physical mixtures of BSA and polymer. Other control experiments investigating the effect of the free radical initiator concentration on the structural integrity of BSA showed that only very high concentrations of the initiator, e.g., ca. 20 times higher than the concentrations used in our experiments, causes aggregation and fragmentation of BSA (Supporting Information, Figure S19-(A)). Additionally, the polymerization of NIPAAm in the presence of nonmodified BSA (without a RAFT agent) did not result in the formation of BSA−poly(NIPAAm) conjugates, as evidenced by the appearance of BSA bands only on PAGE (Supporting Information, Figure S18).

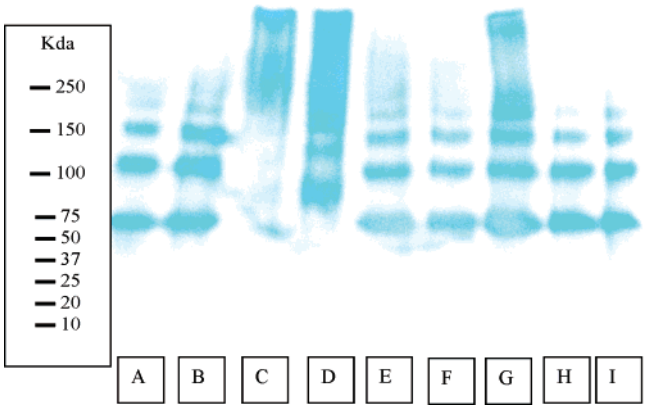

Figure 5. PAGE analysis: (A) BSA−poly(NIPAAm) conjugate after incubation with TCEP (poly(NIPAAm) M_n = 148000 g/mol, PDI = 1.28, respectively); BSA−poly(NIPAAm) conjugates with poly(NIPAAm) M_n and PDI; (B) 148000 g/mol and 1.28, (C) 100000 g/mol and 1.17; (D) 49500 g/mol and 1.13 and (E) 18600 g/mol and 1.08; (F and G) BSA−poly(HEA) conjugate (poly(HEA) M_n = 82000 g/mol, PDI = 1.32) before and after incubation with TCEP, respectively; (H) BSA−macroRAFT agent; (I) BSA.

Hybrid Properties of Well-Defined BSA−Poly(NIPAAm) Conjugates. (i) Bioactivity. It is crucial to assess the effects of the *in situ* RAFT polymerization conditions and polymer conjugation on the biological activity of the protein. It has been shown that bovine serum albumin shows esterase-like activity toward aryl esters such as *p*-nitrophenyl acetate, and this enzyme-like activity requires the conformational integrity of the protein.[90,91] We tested the *p*-nitrophenyl acetate hydrolysis activity of nontreated BSA along with the *in situ* generated BSA−polymer conjugates and BSA treated under varying conditions as control experiments. BSA before and after the

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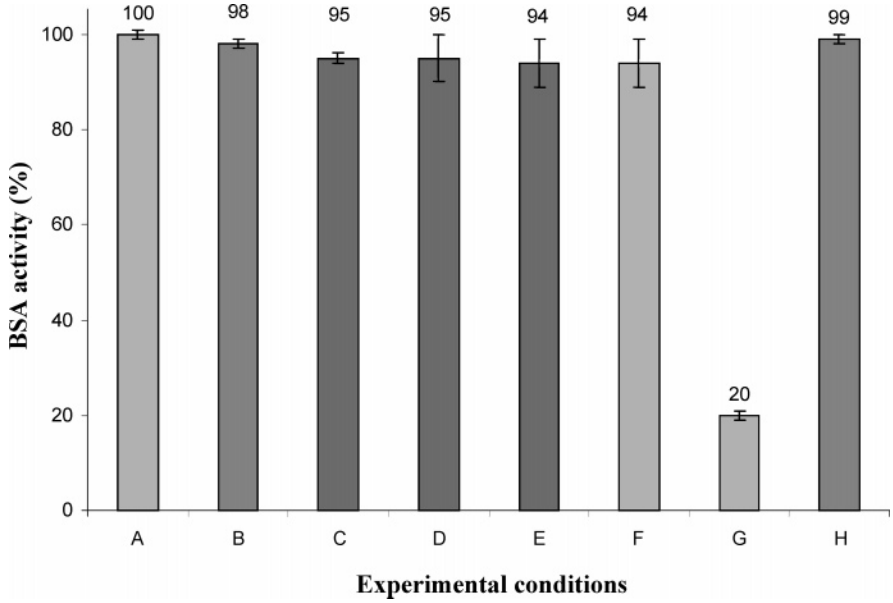

Figure 6. Esterase-like activity of BSA (% of the original activity): (A) nontreated BSA; (B) BSA after conjugation with the RAFT agent; (C) BSA after incubation with free radical initiator (6 mM) in buffer at pH 6.0 at 25 °C for 14 h; (D and E) BSA−poly(NIPAAm) conjugates (mixture of free BSA and conjugate) with poly(NIPAAm) M_n of 46500 and 148000 g/mol, respectively; (F) purified BSA−poly(NIPAAm) conjugate with poly(NIPAAm) M_n of 148000 g/mol (no free BSA); (G) BSA after incubation in buffer at pH 6.0 at 85 °C for 4 h; (H) physical mixture of BSA and poly(NIPAAm) (M_n 140000 g/mol and PDI 1.7). Activity measurements were performed with two different samples in triplicates. The results represent the average of six measurements ± standard deviation. Only F represents the average of two measurements ± standard deviation as it was performed using two different samples with single measurement.

attachment with the RAFT agent showed almost the same activity (Figure 6). The incubation with the free radical initiator only (6 mM) in buffer at pH 6.0 at 25 °C for 14 h caused a slight decrease in the activity of BSA (95 ± 1%, Figure 6). 95% (±5%) of the original activity of BSA was retained after the *in situ* generation of BSA−poly(NIPAAm) conjugates (Figure 6, D and E). The molecular weight of poly(NIPAAm) did not have a significant effect on the protein activity. BSA−poly(NIPAAm) conjugate after separation from the free (non-conjugated) BSA still showed 95 ± 5% of the original BSA activity, showing that the observed activity of the conjugate samples (that were composed of a mixture of free BSA and BSA−polymer conjugates) did not arise only from the free BSA. In a control experiment, the activity assay was found to be not affected by the presence of poly(NIPAAm), i.e. BSA physically mixed with poly(NIPAAm) (M_n 140000 g/mol and PDI 1.7) showed 98% activity (±1%). To check the validity of the activity assay, the activity of BSA after the incubation at 85 °C for 4 h was tested. BSA is known to be denatured upon heating above 65 °C.[92] The activity in this case was 20% (±1%), confirming the validity of the assay to assess the changes in the native conformation of BSA. The effect of the free radical initiator concentration was also tested. The activity of BSA decreased with increasing initiator concentrations (Supporting Information, Figure S19 (B)). However, ca. 80% of the original activity was still retained after the incubation with the initiator at 0.1 M concentration which is ca. 20 times higher than the typical initiator concentration required in a typical free radical polymerization.

(ii) Temperature-Responsive Behavior. Poly(NIPAAm) is a well-known temperature-sensitive polymer. It has a lower critical solution temperature (LCST) at ca. 32 °C in water.[10,93−96] Above the LCST, poly(NIPAAm) becomes dehydrated as a result of an entropy gain due to the release of water molecules around the isopropyl side groups. This temperature-induced dehydration causes the phase separation of poly(NIPAAm) chains from the aqueous solutions. Protein conjugates of poly(NIPAAm) phase-separate at temperatures higher than the LCST of poly(NIPAAm) due to the increased hydrophilicity of the hybrid structure.[82,97−100]

The well-defined BSA−poly(NIPAAm) conjugates are expected to show a uniform, temperature-dependent phase transition behavior exhibiting an LCST higher than the LCST of poly(NIPAAm). The temperature-dependent phase transition behavior of BSA−poly(NIPAAm) conjugates with varying molecular weights was investigated measuring the UV−vis absorbance of the conjugate solutions (0.2 wt %, in distilled water) at 500 nm. The LCST was defined as the temperature at 10% of the maximum absorbance.

As shown in Figure 7 and Table S2, the LCST of the conjugates was higher than poly(NIPAAm) (M_n = 20000 g/mol, PDI: 1.2) and increased with the decrease in the length of poly(NIPAAm) chain conjugated to BSA. The uniformity of the phase transition profiles suggested that the samples were not a mixture of free poly(NIPAAm) and BSA−poly(NIPAAm)

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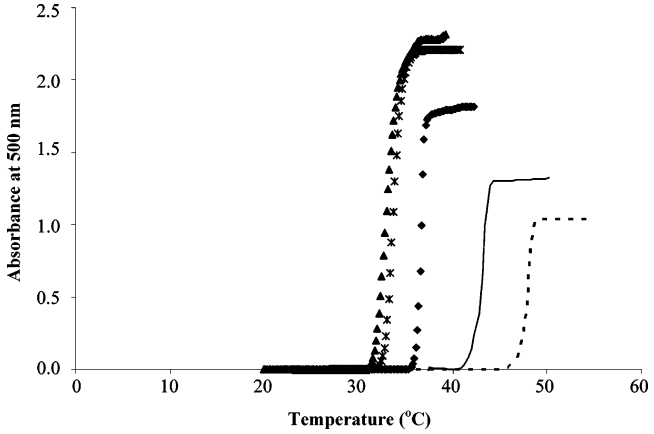

Figure 7. Absorbance of BSA−poly(NIPAAm) conjugate solutions (0.2 wt-%) versus temperature (°C). Conjugates with poly(NIPAAm) M_n and PDI: --- 46500 g/mol and 1.17; — 80000 g/mol and 1.21; ◆ 148000 g/mol and 1.28; Conjugates after reaction with TCEP: ▲ poly(NIPAAm) M_n and PDI 148000 g/mol and 1.28; × 20000 g/mol and 1.20.

conjugate. As the molecular weight of the polymer chain conjugated to the protein decreases, its effect on the hydrophobic/hydrophilic balance of the hybrid structure becomes less profound while the effect of BSA, temperature nonresponsive and hydrophilic component of the hybrid, becomes dominant. Hence, LCST of the conjugates increases with the decrease in the conjugated poly(NIPAAm) molecular weight. The maximum absorbance values of the conjugates upon thermoprecipitation decreased with the decrease in the molecular weight of the conjugated polymer. This was attributed to the formation of less condensed aggregates due to the dominancy of the hydrophilic and nonresponsive component, i.e., BSA in the low molecular weight hybrid structure.[69,82,101]

The LCST of BSA−poly(NIPAAm) conjugate (molecular weight of poly(NIPAAm): 148000 g/mol, PDI: 1.28) was shifted from 35 °C to 32 °C after the reaction with TCEP. This result indicated that the polymer chains generated were conjugated to the protein via reducible bonds and also the higher LCST value of the conjugate compared to the LCST of poly(NIPAAm) was due to the attached BSA.

(iii) Temperature-Induced Aggregation. The change in the hydrodynamic diameter of BSA−poly(NIPAAm) conjugates with temperature was tested using dynamic light scattering (Figure 8). For temperatures below the LCST, all components, i.e., BSA and poly(NIPAAm), are hydrophilic, and there is no driving force for aggregation. Therefore, the size for both poly(NIPAAm) and BSA−poly(NIPAAm) conjugates (i.e., z-average hydrodynamic diameter), indicated the presence of unimers only (Supporting Information, Figure S21). It was noted that the hydrodynamic diameter of BSA−poly(NIPAAm) conjugate (14−16 nm) was higher than that of poly(NIPAAm) (8−10 nm) (Supporting Information, Figure S21). For temperatures above the LCST, particle formation was observed (by cumulative method), which was indicated by the significant increase in the scattering intensity.[82,100] The LCST value could be defined from the temperature value at which the formation of aggregates was observed. The LCSTs obtained by light scattering were in good agreement with the ones obtained by turbidity measurements via UV−vis spectrophotometer. Above

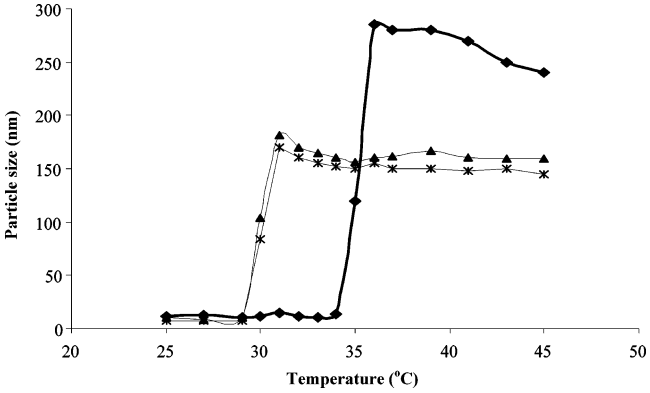

Figure 8. Hydrodynamic diameter versus temperature. ◆ BSA−poly(NIPAAm) conjugate with poly(NIPAAm) M_n = 148000 g/mol, PDI: 1.28, and ▲ after reaction with TCEP, × poly(NIPAAm), M_n = 20000 g/mol, PDI: 1.20.

the LCSTs, the conjugates exhibited particle sizes of 250−300 nm, while poly(NIPAAm) exhibited much smaller particle sizes (i.e., 150 nm) for the same conditions. The difference in the particle size of the conjugates and poly(NIPAAm) was attributed to the higher hydrophilicity of BSA−polymer conjugates compared to poly(NIPAAm) due to the presence of the conjugated BSA, which leads to the formation of more hydrated particles upon aggregation. The particle size of the conjugates shifted to ca. 180 nm after the reaction with TCEP, confirming again that poly(NIPAAm) attachment to BSA was via reducible linkages.

Conclusion

The RAFT polymerization of NIPAAm and HEA was achieved in completely aqueous medium at room temperature using a BSA−macroRAFT agent. The evolution of the monomer conversions and the molecular weights of the polymers formed during the *in situ* polymerization showed the characteristics of the RAFT mechanism. The results revealed the *in situ* formation of BSA−polymer conjugates without any detectable free polymer formation. The efficiency of BSA−macroRAFT agent to generate BSA−polymer conjugates was found to be ca. 1. The structural integrity and the conformation-related activity of BSA were found to be not affected by the polymerization conditions and the conjugation of the polymer chain. BSA−poly(NIPAAm) conjugates showed hybrid temperature-dependent phase separation and particle formation behavior.

Biotechnology and biomedicine applications would benefit from the generation of well-defined, monodisperse biomolecule-polymer conjugates. Our results show overall that the use of the RAFT technique for preparation of protein−polymer conjugates is a versatile and highly efficient approach in terms of excellent control over polymerization without the need for large quantity of RAFT agent modified biomolecules and elimination of the postpolymerization purification steps. Also providing the variety in monomers that can be polymerized by RAFT, the utilization of the RAFT technique is a promising step for generation of well-defined, homogeneous protein−polymer conjugates in one-step without the need for postpolymerization purifications. Our future efforts will be focused on

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investigation of the applicability of the RAFT-generated protein particles as carriers for biological systems.

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Supporting Information Available: Experimental section, synthesis, and characterization of RAFT agent (^{1}H NMR and ESI spectrum), ^{1}H NMR and aqueous SEC of BSA−macroRAFT agent, Ellman's assay results, polymerization data of NIPAAm and HEA in the presence of BSA−macroRAFT agent (aqueous SEC traces and ^{1}H NMR of crude BSA−poly(NIPAAm) conjugates), ^{1}H NMR of isolated BSA−poly(NIPAAm) conjugates, deconvolution results, cleavage of polymer from protein, measurement of enzyme-like activity of BSA−polymer conjugates, LCST and particle size measurements, complete references 7 and 99. This material is available free of charge via the Internet at http://pubs.acs.org.

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