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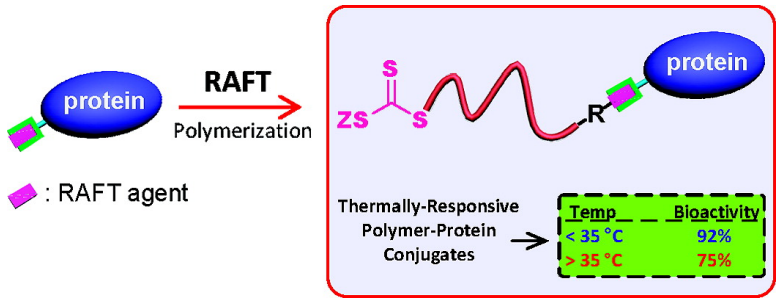
J. Am. Chem. Soc., **2008**, 130 (34), 11288-11289 • DOI: 10.1021/ja804495v • Publication Date (Web): 30 July 2008



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Temperature-Regulated Activity of Responsive Polymer−Protein Conjugates Prepared by Grafting-from via RAFT Polymerization

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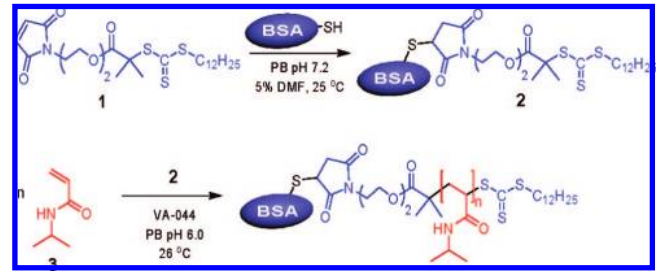
Covalent modification with synthetic polymers is an effective method to tune the solubility, biocompatibility, stability, and activity of proteins, peptides, and nucleic acids.[1] Bioconjugation with stimuli-responsive polymers is particularly useful because the responsive nature of the polymer can be conferred to the substrate to which it is attached. We are primarily interested in preparing "smart" polymer−protein conjugates[2] to facilitate applications in drug delivery, enzymatic catalysis, biosensing, bioseparation, etc. Realization of these applications requires techniques that streamline synthesis of well-defined responsive polymers and their immobilization onto biomacromolecules.

Polymer−protein bioconjugates are generally synthesized by reaction of preformed end-functional polymers with proteins.[3] Purification of the targeted polymer−protein bioconjugates prepared by this *grafting-to* methodology can be tedious, and depending on the protein functionality targeted for conjugation, the number and location of grafted polymer chains can be difficult to ascertain. An alternative conjugation strategy is to modify the protein with an initiating group and subsequently polymerize by grafting directly from the protein. This *grafting-from* approach simplifies purification because an unreacted monomer is easily removed, and access to high molecular weight conjugates is facilitated since the sterics associated with coupling a large polymer and protein are avoided. Employing this strategy via atom transfer radical polymerization[4] has proven particularly valuable.[5] The adoption of another controlled radical polymerization technique, reversible addition−fragmentation chain transfer (RAFT),[6] for the preparation of bioconjugates has been considerably more gradual,[7] despite the absence of a metal catalyst, ready amenability to nondenaturing aqueous media,[8] extensive monomer class applicability, and vast functional group tolerance. Very recently, Bulmus and Davis et al. utilized RAFT polymerization for *in situ* formation of disulfide-linked bovine serum albumin (BSA)−poly(ethylene glycol acrylate)[9] and BSA−poly(*N*-isopropylacrylamide)[10] (PNIPAM) conjugates in aqueous medium in the presence of a BSA−macro chain transfer agent (CTA). The RAFT CTA (ZC(=S)S−R) was immobilized to the protein through its "Z-group," leading to a controlled polymerization mediated by a process involving a combination of effects associated with the grafting-to and -from approaches.

To build upon the success of RAFT polymerization for preparing stable well-defined bioconjugates, it is advantageous to also design macroCTAs with the R-group of the CTA anchored to the biomacromolecule surface.[11] In such a case, the thiocarbonylthio moiety is distal to the protein and is potentially more readily accessible for chain transfer with propagating chains in solution, a reaction the facility of which is required for molecular weight control. Additionally, because the relatively labile thiocarbonylthio moiety would reside on the free end group of the immobilized polymer and not be responsible for the conjugation linkage, the conjugates prepared in this manner should demonstrate increased stability and the potential for transformation into thiol groups for subsequent surface immobilization or labeling. Herein, a protein−macroRAFT agent of this type was prepared and subsequently employed for aqueous RAFT polymerization of *N*-isopropylacrylamide (NIPAM, **3**) to obtain conjugates with activity reliant upon the responsive nature of the immobilized polymer.

Scheme 1. Modification of BSA with a Maleimide-Functionalized CTA and Grafting-from via RAFT Polymerization of NIPAM



A protein−macroCTA (**2**) was synthesized by reaction of a maleimide-functionalized trithiocarbonate (**1**) with a free sulfhydryl cysteine residue (Cys-34) of BSA (Scheme 1). This reaction occurs readily and is specific for thiol groups at ambient pH. Ellman's analysis indicated near-quantitative functionalization of Cys-34 by comparing the number of free cysteine residues present before (45 mol%) and after (∼0 mol%) conjugation with the RAFT agent. Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy provided direct evidence of efficient protein functionalization with the CTA group (Supporting Information).

The versatility of RAFT allowed polymerization of NIPAM (300 mM) with **2** (0.30 mM) and water-soluble initiator 2,2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044, 1.5 mM) at 26 °C directly in phosphate buffer (PB, pH 6). Conversion of NIPAM determined gravimetrically and by ^{1}H NMR spectroscopy was in excellent agreement and suggested that, after an initial induction period, a constant concentration of radicals was present up to high conversion (Figure 1a). Aqueous size exclusion chromatography (SEC) confirmed the presence of polymer−protein conjugates with larger sizes than the original BSA−macroCTA (Figure 1b). As expected, a fraction of unconjugated protein remained since the BSA originally subjected to functionalization with **1** contained only 45 mol% available thiols at Cys-34, as is commonly observed for adventitiously oxidized commercial batches of BSA. However, because the presence of immobilized PNIPAM led to the conjugates demonstrating responsive behavior with a lower critical solution temperature (LCST) of 35 °C, the mixture of conjugate and free BSA was easily separated by thermal precipitation[10] of the responsive conjugates at 40 °C.

The robust thioether linkage between polymer and protein complicated selective cleavage of the chains for molecular weight characterization. However, treatment of conjugate samples taken at various monomer conversions with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) for 5 days at room temperature led to near-quantitative protein decomposition.[9] After dialysis to remove low molecular weight

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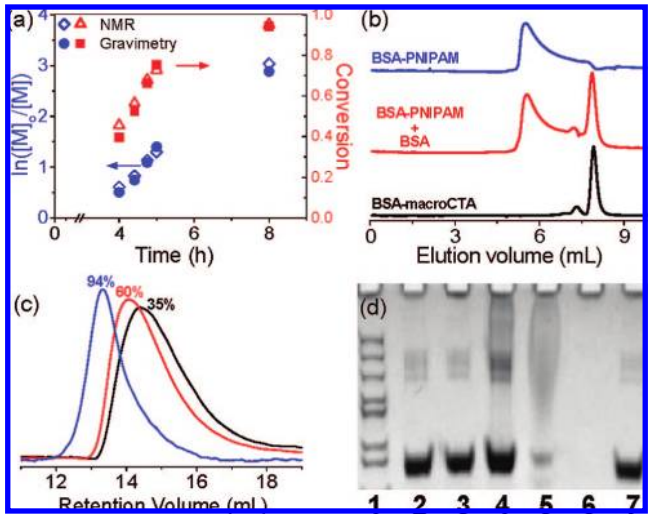

Figure 1. Results from the RAFT polymerization of NIPAM with BSA−macroCTA (**2**): (a) Kinetics; (b) SEC traces for BSA−macroCTA (**2**), BSA−PNIPAM conjugate (94% conv.), and BSA−PNIPAM after thermal precipitation; (c) SEC traces of cleaved PNIPAM after treating BSA−PNIPAM conjugates with TCEP; and (d) PAGE results (lane 1: MW markers, 2: BSA, 3: BSA−macroCTA (**2**), 4: BSA−PNIPAM with free BSA, 5: BSA−PNIPAM after thermal precipitation, 6: PNIPAM (M_n = 48 kg/mol), 7: BSA+PNIPAM (M_n = 48 kg/mol) physical mixture).

polypeptide fragments, SEC of the "cleaved" polymer verified an increase in molecular weight with conversion, as expected for the RAFT process (Figure 1c). Molecular weight control was good; for example, at 94% conversion an $M_{n,theory}$ of 239 000 g/mol agreed well with the experimental molecular weight 234 000 g/mol (M_w/M_n = 1.38), calculated from the macroCTA concentration determined using the degree of functionalization from Ellman's analysis.

Further evidence of successful polymer−protein conjugation was obtained by polyacrylamide gel electrophoresis (PAGE) (Figure 1d). The native BSA (lane 2) and BSA−macroCTA (**2**) (lane 3) yielded near identical bands at ~67 000 and 134 000 g/mol (dimer). Owing to its significantly increased size, the BSA−PNIPAM conjugate appeared as a high molecular weight band (lane 4). Unconjugated BSA was again observed, but efficient removal during thermal precipitation was evidenced by its substantial diminishment in lane 5. A physical mixture of protein and PNIPAM resulted in bands only for BSA, indicating that the protein and polymer do not form conjugates via simple physical interactions. Polymer alone was not visible (lane 6), offering further evidence that the bands appearing in the bioconjugate lane were indeed the result of modified protein.

Circular dichroism indicated retention of the protein secondary structure in both the BSA−macroCTA and the BSA−PNIPAM conjugate. The modified protein derivatives were also tested for their ability to hydrolyze 4-nitrophenylacetate, an esterase-like activity known to be dependent on the structural integrity of the protein.[12] The BSA−macroCTA and BSA−PNIPAM conjugates demonstrated >90% retention of activity as compared to native BSA (Figure 2a). Particularly interesting was that the conjugate purified by thermal precipitation retained 92 ± 4% of its activity at 25 °C, but when the assay was conducted at 40 °C, above the LCST of the attached PNIPAM, the activity was reduced to 75 ± 2%. Thus, the responsive nature of the polymer allowed thermal regulation of the biological behavior of the protein.[13] Several heating/cooling cycles demonstrated the activity switching was highly repeatable, offering evidence the protein was not denatured during heating (Figure 2b).

These results indicate protein modification with a RAFT CTA and subsequent room temperature polymerization in aqueous media is a viable means to prepare responsive polymer−protein conjugates. Using

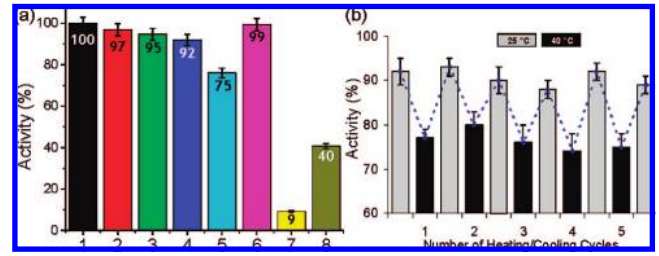

Figure 2. (a) Activity of (1) BSA, (2) BSA−macroCTA, (3) BSA−PNIPAM (free BSA present) with conjugated polymer of 234 000 g/mol, (4) BSA−PNIPAM thermal precipitate, (5) BSA−PNIPAM thermal precipitate at 40 °C assay temperature (with respect to BSA at 40 °C), (6) BSA+PNIPAM physical mixture, (7) PNIPAM, (8) BSA after incubation at 75 °C for 3 h. All assays were conducted with identical [BSA]. (b) Activity of BSA−PNIPAM thermal precipitate during thermal cycling between 25 and 40 °C.

the approach of thiol-maleimide immobilization via the CTA fragment responsible for initiating new chains, high molecular weight and reductively stable conjugates are accessible without extensive purification procedures and without adverse effects on the protein structure. The responsive nature of the immobilized polymer facilitates conjugate isolation and also allows environmental modulation of bioactivity. This approach should be extendable to the aqueous synthesis of a variety of other smart polymer−protein conjugates without metal catalysts, organic cosolvents, or high temperatures.

Acknowledgment. We thank DARPA (HR0011-06-1-0032) and Oak Ridge Associated Universities for financial support and Dr. Jamie Messman and Dr. Deanna Pickel for the MALDI-TOF MS analysis. A portion of this research (MALDI-TOF MS) at Oak Ridge National Laboratory's Center for Nanophase Materials Sciences was sponsored by the Scientific User Facilities Division, Office of Basic Energy Sciences, U.S. Department of Energy.

Supporting Information Available: Additional results and detailed experimental/analytical details. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA804495V