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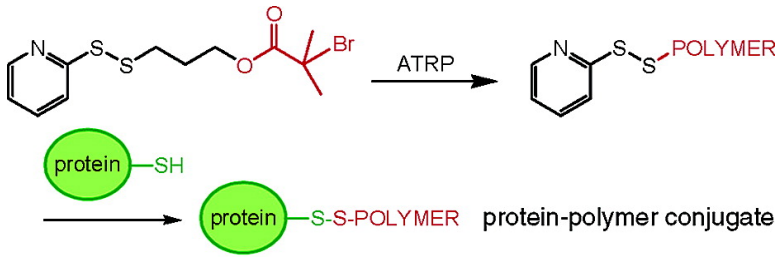

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Cysteine-Reactive Polymers Synthesized by Atom Transfer Radical Polymerization for Conjugation to Proteins

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Protein−polymer conjugates are widely used in medicine and biotechnology.[1] They are typically prepared by reaction of semi-telechelic polymers with amino acid side chains.[2] To achieve site-specific conjugation, cysteine residues not participating in disulfide bonds are often targeted.[3] If a protein does not naturally contain these "free" cysteines, genetic engineering methodologies can be used to strategically place them in the amino acid sequence.[1,3] Thiol-reactive polymers with terminal maleimides, vinyl sulfones, iodoacetamides, and activated disulfides have been synthesized for conjugation to proteins.[4] However, because the reactions involve postpolymerization modification of chain ends, the schemes typically require many synthetic steps.

The use of an initiator for polymerization that also reacts directly with sulfhydryl groups would allow the synthesis of functional polymers amenable to coupling to proteins without further reaction or activation steps. Poly(ϵ-caprolactone)s functionalized with thioester end groups have been synthesized using dimethylaluminum benzylthiolate as an initiator for ring-opening polymerization of the lactone.[5] The polymers react with N-terminal cysteine residues by native chemical ligation. However, to our knowledge, this strategy has not yet been employed to prepare polymers capable of reacting with any cysteine residue in an amino acid sequence. Herein, we report the synthesis of polymers using an activated disulfide-functionalized initiator and demonstrate the direct formation of protein−polymer conjugates without the need for any postpolymerization modification.

Recently, it has been demonstrated that well-defined polymer chains are important for resultant bioconjugate properties, including bioactivity and self-assembly.[6] Atom transfer radical polymerization (ATRP) results in polymers with narrow molecular weight distributions[7] and is a robust methodology tolerant of many functional groups.[8] Thioether-[9] and alkyl disulfide-containing[10] initiators have been successfully employed in ATRP. Treatment of the resulting polymers with a reducing agent such as dithiothreitol (DTT) yielded mercapto-terminated polymers. Therefore, pyridyl disulfide-functionalized initiator **1** was prepared and used for CuBr/2,2′-bipyridine (bipy)-mediated ATRP of 2-hydroxyethyl methacrylate (HEMA, Scheme 1). Pyridyl disulfides are known to undergo direct coupling with free cysteines under mild conditions releasing 2-pyridinethione.[3b] HEMA was chosen because the ATRP polymerization of this monomer is controlled, and pHEMA is widely used in biomaterial applications.[11]

Polymerization of HEMA with the activated disulfide initiator was explored using a 50/1 initial HEMA to **1** ratio in deuterated methanol at 23 °C. Typical kinetic and evolution of polymer molecular weight versus conversion data are depicted in Figure 1. The semilogarithmic kinetic plot is linear (Figure 1a), suggesting a constant concentration of growing radicals to 95% conversion. In addition, the number average molecular weight (M_n) increases linearly with conversion and the polydispersity indices (PDIs)

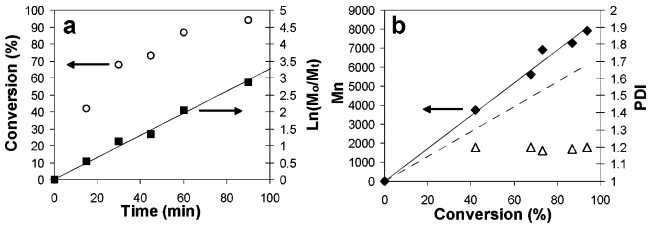
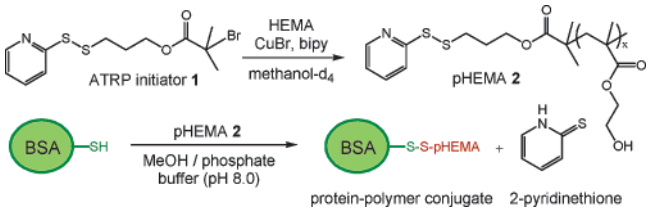


Figure 1. ATRP of HEMA in methanol from initiator **1**. $[HEMA]_0:[\mathbf{1}]_0:[CuBr]_0:[bipy]_0 = 50:1:1:2$. (a) Kinetic plot. (b) Experimental M_n (from ^{1}H NMR) and PDI (from GPC) vs conversion. The dashed line represents the theoretical molecular weight.

Scheme 1. Reaction Scheme for Polymer Synthesis and Conjugation



remain close to 1.2 throughout the polymerization (Figure 1b). The actual molecular weight determined by ^{1}H NMR is higher than the theoretical molecular weight, which may indicate low initiator efficiency. Nevertheless, polymers with different molecular weights were readily accessible by changing the initial monomer-to-initiator ratio (Table 1). At higher conversions, a slight asymmetry in the gel permeation chromatography (GPC) traces and/or a small shoulder at higher molecular weights was detected. Similar observations were reported by Matyjaszewski et al. for alkyl disulfide ATRP initiators[10] and can be ascribed to side reactions such as chain transfer or coupling. Improved GPC traces were obtained by employing CuCl/bipy as the catalyst to slow the polymerization and reduce side reactions.

The presence of the activated disulfide is critical for resulting applications. Since chain transfer to initiator or chain ends would lower the disulfide content of the polymers, careful inspection of end-group functionality was carried out. Comparison of the integration of the proton ortho to the pyridyl nitrogen (A, Figure 2) with the methylene protons adjacent to the disulfide (E, Figure 2) for all the samples revealed that 86−91% of the chains were substituted with a pyridyl disulfide group (Table 1).[12] Standard thione release studies were employed to verify the results.[13] The polymer samples were treated with DTT or cysteine, and the concentration of released 2-pyridinethione, which absorbs at 343 nm, was determined using UV spectroscopy. The percent of polymers with activated end groups by either reduction or reaction with cysteine was greater than 80%. All together, these results indicate that although minimal chain transfer reactions are likely

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Table 1. Characteristics of the Pyridyl Disulfide Polymers[a]

[HEMA]$_0$/[**1**]$_0$	time (min)	conversion (%)	M_n (theory)	M_n (^{1}H NMR)	PDI (GPC)	% end group[b]
10	60	>95	1300	3900	1.20	91
50	90	95	6110	7910	1.20	91
100	180	91	11 390	15 700	1.25	86

[a]Polymerization conditions: [HEMA]$_0$ = 50 v/v %, CD_3OD, 23 °C, [**1**]$_0$:[CuBr]$_0$:[bipy]$_0$ = 1:1:2. [b]Calculated from ^{1}H NMR spectra.[12]

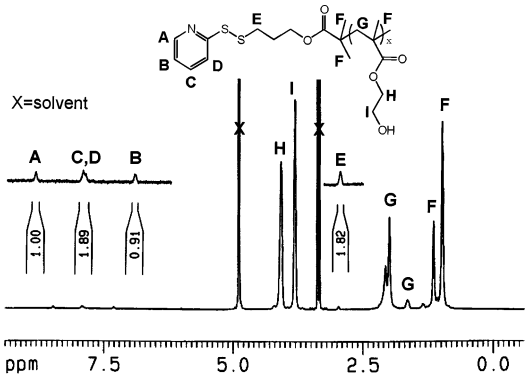

Figure 2. Exemplary ^{1}H NMR spectrum (500 MHz) in CD_3OD of pHEMA obtained from initiator **1** ([HEMA]$_0$:[**1**]$_0$:[CuBr]$_0$:[bipy]$_0$ = 50:1:1:2).

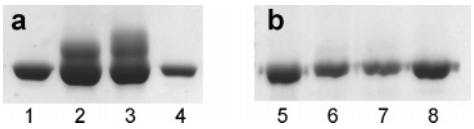

Figure 3. SDS−PAGE in nonreducing (a) and reducing (b) conditions. Lanes 1, 5: BSA. Lanes 2, 6: BSA + pHEMA **2** ([HEMA]$_0$/[**1**]$_0$ = 50). Lanes 3, 7: BSA + pHEMA **2** ([HEMA]$_0$/[**1**]$_0$ = 100). Lanes 4, 8: BSA + pHEMA control.

occurring, the majority of polymer chains are still functionalized with the activated disulfide group.

The as-synthesized semitelechelic polymers should react with proteins to form bioconjugates. To demonstrate this, polymers were incubated with bovine serum albumin (BSA), a globular protein naturally displaying a free cysteine on residue 34. The couplings were performed by adding solutions of pHEMA **2** in methanol to BSA dissolved in phosphate buffer at pH = 8.0 (Scheme 1) and incubating the mixture at room temperature for 30 min. The formation of the conjugates was verified by gel electrophoresis (Figure 3a). The gel clearly shows a shift of the conjugates (lanes 2 and 3) compared to BSA alone (lane 1). The presence of unreacted BSA can be attributed to the partial oxidation of the Cys-34 of commercial batches of BSA, as documented in the literature.[14] Ellman's assay[3b] performed on the conjugate as well as on the native BSA confirmed that, while only 45−50% of the Cys-34 in the starting BSA was available for conjugation, less than 6% thiols were still present after incubation with pHEMA **2**. To exclude the possibility that the changes in the gel result from a physical mixture of the BSA and polymer, BSA was incubated with nonreactive pHEMA synthesized using ethyl 2-bromoisobutyrate as the initiator. As expected, the polymer does not cause a shift of the BSA band (lane 4). Additionally, after the samples were treated with DTT, the conjugates were no longer detected (Figure 3b). Although this result does not confirm that the anchoring point of the polymer is the cysteine residue 34, it does demonstrate that conjugation occurs through a reversible disulfide bond.

In conclusion, we have developed a new, straightforward approach to the preparation of thiol-reactive polymers for conjugation to proteins. pHEMAs with low polydispersity indices functionalized at one end with activated disulfides were prepared by ATRP using an initiator modified with a pyridyl disulfide group. Conjugation of the polymers to BSA was readily achieved and verified by gel electrophoresis and Ellman's assay. This strategy is applicable for the preparation of a wide variety of polymer−protein conjugates without the need for postpolymerization modification of the polymers.

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Supporting Information Available: Materials and methods and GPC traces of the end-functional polymers (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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