Direct Synthesis of Well-Defined Heterotelechelic Polymers for Bioconjugations

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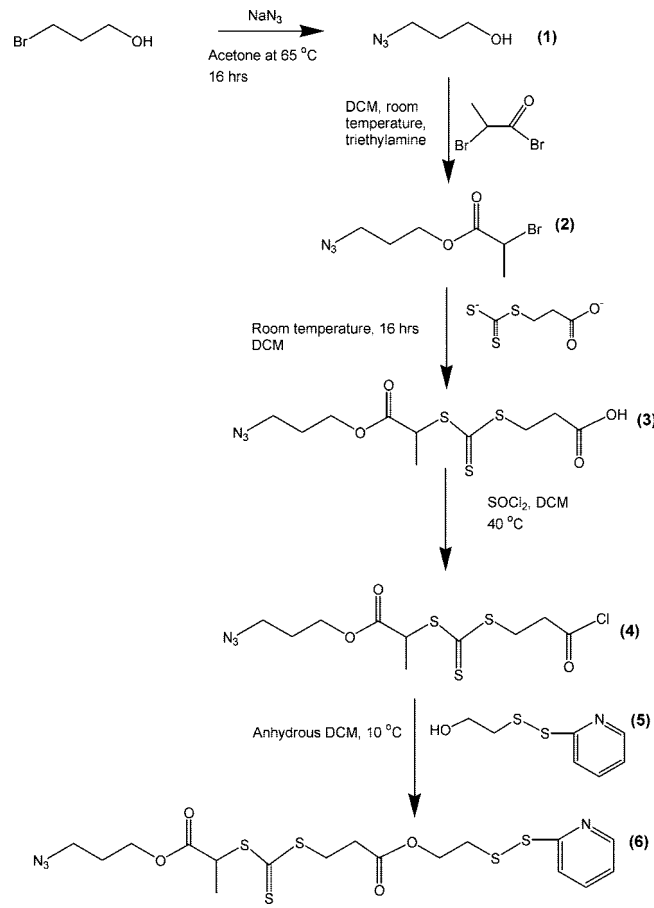
ABSTRACT: Direct synthesis of well-defined heterotelechelic polymers having functional groups allowing the chemoselective bioconjugations would be desirable to enhance the versatility of polymers for bioconjugations and bio-related applications. Considering this, well-defined α-azide, ω-dithiopyridine polymers were synthesized in one step via the reversible addition−fragmentation chain transfer (RAFT) polymerization. The telechelic functionality (i.e., mole ratio of the ω-dithiopyridine to the α-azide end group) of polymers was above 0.90, indicating the efficient generation of well-defined heterotelechelic polymers. The heterotelechelic functionality for chemoselective bioconjugations was tested by reacting α-azide, ω-dithiopyridine poly(NIPAAm) with model biomolecules, i.e., biotin/avidin, glutathione, and bovine serum albumin, via click and thiol−disulfide exchange chemistries. Near-stoichiometric conjugation with biomolecules indicated high functionality of the polymer end groups.

Introduction

Bioconjugates of vinyl polymers have been increasingly used in biomedicine, biotechnology, and nanotechnology.[1–4] In general, they have been prepared using semitelechelic polymers having one functional end group which could be used for conjugation directly or after chemical modifications.[5–7] While the entry of controlled/living radical polymerization (CLRP) techniques to the bioconjugation field has enabled the in-situ synthesis of bioconjugates of polymers[8–13] as well as the direct synthesis of well-defined semitelechelic polymers suitable for conjugation to biomolecules without the need for postpolymerization modifications,[9,14–23] they have also brought the possibility of synthesizing in one step well-defined telechelic polymers. To date, while a large number of homotelechelic polymers[18,24–32] which possess the same functionality at both chain ends have been synthesized directly via the CLRP techniques, only a few heterotelechelic polymers that possess different reactive groups suitable for chemoselective bioconjugations[27,33–38] could be generated only after postpolymerization modifications. It is clear that the direct synthesis of well-defined heterotelechelic polymers having functional groups allowing the selective bioconjugations would be desirable to enhance the versatility of polymer bioconjugates and hence improve their bio-related applications.

Considering this, we have synthesized in one-step α-azide, ω-dithiopyridine heterofunctional, well-defined polymers via the reversible addition−fragmentation chain transfer (RAFT) polymerization[39–42] for chemoselective conjugation to biomolecules (Scheme 1). Dithiopyridine and azide groups have been proven to be suitable for selective bioconjugation chemistries, i.e., dithiopyridine for selective exchange reaction with free thiol containing (or engineered) biomolecules[12,15,43] and azide for click reaction[44–46] with alkyne engineered biomolecules.[4,37,47–56] A new RAFT agent bearing an azide and a dithiopyridine group at R and Z fragments, respectively, was first synthesized and used to control the polymerization of styrene, *N*-isopropylacrylamide (NIPAAm), and oligo(ethylene glycol) acrylate (OEGA). The kinetic study of polymerizations and ^{1}H NMR and mass spectroscopy characterization of the purified polymers confirmed the efficient generation of well-defined heterotelechelic polymers. α-Azide functionality was first tested by reacting α-azide, ω-dithiopyridine poly(NIPAAm) with alkyne-modified biotin.

Scheme 1. Synthesis of the RAFT Agent 3-Azidopropyl-2-(2-(2-pyridyldisulfanyl)ethoxycarbonyl)ethyl dithiocarboxysulfanyl Propionate



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α-Biotin, ω-dithiopyridine poly(NIPAAm) was further reacted with thiol-containing model biomolecules, i.e., a tripeptide, reduced glutathione, and also a protein, bovine serum albumin (BSA). End-group modifications were characterized by ^{1}H NMR, FTIR, and UV−vis spectrometry, HABA assay, and gel permeation chromatography (GPC). Heterobiofunctional poly(NIPAAm) was further modified with biotin's affinity protein,[57] i.e., avidin, to form α-avidin, ω-BSA-functionalized poly(NIPAAm). The formation of the affinity complex of avidin with the BSA polymer conjugate was characterized by GPC. The results showed that ω-dithiopyridine and α-biotin functionalities of poly(NIPAAm) could be used efficiently for direct generation of heterofunctional well-defined bioconjugates via selective covalent and affinity binding strategies.

Experimental Section

Materials. All reagents were purchased from Aldrich at the highest purity level available and used without further purification unless noted. *N*-Isopropylacrylamide (NIPAAm) (97%) was recrystallized twice from hexane prior to use (mp = 64 °C). 2,2′-Azobis(isobutyronitrile) (AIBN) was recrystallized twice from methanol prior to use. Oligo(ethylene glycol) acrylate (OEG-A) (number-average molecular weight M_n = 450 g/mol, PDI = 1.04) was run through an alumina column to remove the inhibitor prior to use. Styrene was distilled over CaH_2 prior to use. The other materials used and their characteristic details are given in the Supporting Information.

Synthesis of the RAFT Agent. *Step 1: Synthesis of 3-Azido-1-propanol (Product* **1***).* 3-Bromopropanol (5 g, 36 mmol) and sodium azide (3.83 g, 59 mmol) were dissolved in a mixture of acetone/water (60 mL/10 mL), and the resulting solution was refluxed overnight. Acetone was removed under reduced pressure, 50 mL of water was added, and the mixture was extracted by DCM (3 × 100 mL). The organic phase was collected and dried over $NaSO_4$. After removal of the solvent under reduced pressure, 3-azido-1-propanol (**1**, Scheme 1) was isolated as colorless oil (2.82 g, yield 77%). ^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm from TMS): 1.76 (2H, q, J = 6.3 Hz, −CH_2−CH_2−CH_2OH), 3.18 (1H, s, CH_2−OH), 3.38 (2H, t, J = 6.6 Hz, −CH_2−N_3), 3.67 (2H, t, J = 6.3 Hz, −CH_2−OH). The spectrum can be seen in Figure S1 (Supporting Information). ^{13}C NMR (75 MHz, $CDCl_3$): 31.32 (−CH_2−CH_2−CH_2O−), 48.27 (CH_2−N_3), 59.48 (−CH_2−OH). FT-IR (cm^{-1}): 3331, 2945, 2881, 2087, 1455, 1258, 1044, 955, 900.

Step 2: Synthesis of 3-Azidopropyl-2-bromopropionate (Product **2***).* A solution of 2-bromopropyl bromide (8.42 g, 39.00 mmol) in dry dichloromethane (25 mL) was added dropwise to a solution of 3-azido-1-propanol (3.00 g, 29.64 mmol) and dry triethylamine (6.11 mL, 4.44 mmol) in dry dichloromethane at 0 °C. After complete addition, the reaction mixture was stirred at room temperature for 2 h. The excess of acid bromide was quenched by addition of water. The formed triethylammonium bromide salt was filtered off. The crude solution was washed twice with acidic water at pH 4, twice with $NaHCO_3$ solution at pH 9, and twice with distilled water. The organic phase was dried with sodium sulfate. The product was purified by silica gel flash chromatography (hexane/ethyl acetate, 5/1). After the removal of the solvents, the product (**2**, Scheme 1) was isolated as colorless oil (5.5 g, yield 80%). ^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm from TMS): 1.78 (3H, d, −CH_3), 1.94 (2H, q, J = 6.1 Hz, −CH_2−CH_2−CH_2−), 3.38 (2H, t, J = 6.6 Hz, −CH_2−N_3), 4.17 (2H, t, J = 6.3 Hz, −CH_2−O−), 4.27 (1H, CH). The spectrum can be seen in Figure S1 (Supporting Information). ^{13}C NMR (75 MHz, $CDCl_3$): 22.22 (CH_3), 27.82 (−CH_2−CH_2−CH_2O−), 40.10 (CH_2O), 48.27 (CH_2−N_3), 63.10 (−CH−), 170.10 (C═O). FT-IR (cm^{-1}): 2979, 2930, 2087, 1735, 1446, 1379, 1334, 1258, 1220, 1155, 1097, 1061, 1029, 763, 675.

Step 3: Synthesis of 3-Azidopropyl-2-(2-carboxyethyl)dithiocarboxysulfanyl Propionate (Product **3***).* 2-Mercaptopropionic acid was dissolved in diethyl ether; triethylamine was added at 0 °C. Carbon disulfide was then added slowly. The solution was stirred at room temperature for 14 h to yield an orange precipitate. The solvent was removed under vaccum. The crude product was precipitated two times in diethyl ether, which yielded an orange viscous product. This product was used without further purification. ^{1}H NMR confirmed the structure expected. ^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm from TMS): 1.2 (18H, t, CH_3 of TEA), 2.5 (2H, t, −CH_2−S−), 2.7 (2H, t, −CH_2−COO^-), 2.9 (12H, q, N−CH_2− of TEA). 3-Azidopropyl-2-bromopropionate (product **2**, 5.00 g, 21.6 mmol) was dissolved in DCM (50 mL) and stirred with a 2-fold excess of dithiocarboxysulfanylpropionic acid salt (15.94 g, 43.2 mmol) for 16 h at room temperature. The triethylammonium bromide was filtered and washed several times with DCM (50 mL). The product was purified by extraction twice with deionized water (20 mL) and three times with acidic water (pH 2). The organic phase was dried on sodium sulfate (Na_2SO_4) and then rotary evaporated. The final yellow oil was purified by silica gel column (chloroform/methanol: 10/1, vol %) (yield 70%). ^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm from TMS): 1.59 (3H, d, −CH_3), 1.94 (2H, q, J = 6.1 Hz, −CH_2−CH_2−CH_2−), 2.81 (2H, t, J = 6.1 Hz −CH_2−S−), 3.41 (2H, t, J = 6.6 Hz, −CH_2−N_3), 3.60 (2H, t, J = 6.2 Hz, −CH_2−CO_2H), 4.17 (2H, t, J = 6.3 Hz, −CH_2−O−), 4.78 (1H, quartet, CH), 10.0 (1H, s, CO_2H). The spectrum can be seen in Figure S1 (Supporting Information). ^{13}C NMR (75 MHz, $CDCl_3$): 20.14 (CH_3), 29.82 (−CH_2−CH_2−CH_2O−), 40.10 (CH_2O), 48.27 (CH_2−N_3), 62.5 (−CH−), 170.10 (C═O), 179.2 (−CO_2H), 222.10 (C═S). FT-IR (cm^{-1}): 2979, 2930, 2087, 1730, 1715, 1446, 1379, 1334, 1258, 1220, 1155, 1097, 1061, 1029, 763, 675. ESI-MS: 360.6 (Na^+) (theoretical value = 360.45). This RAFT agent was found to decompose in the mass spectrometer before the ionization. Molecular peak and assigned fragments were detected, but at concentrations lower than expected.

Step 4: Chlorination of 3-Azidopropyl-2-(2-carboxyethyl)dithiocarboxysulfanyl Propionate. Product **3** was dissolved in dried DCM (50 mL) and stirred with a 5-fold excess of thionyl chloride for 16 h at room temperature. The solvent and the excess of thionyl chloride was evaporated under vacuum (P = 20 mmHg, at 35 °C). The product (**4**, Scheme 1) was analyzed by ^{1}H NMR directly without further purification. The absence of carboxylic acid group (absence of the proton signal centered at 10.0 ppm attributed to −COOH) confirmed the quantitative reaction without any degradation. ^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm from TMS): 1.59 (3H, d, −CH_3), 1.94 (2H, q, J = 6.1 Hz, −CH_2−CH_2−CH_2−), 2.81 (2H, t, J = 6.1 Hz, −CH_2−S−), 3.41 (2H, t, J = 6.6 Hz, −CH_2−N_3), 3.70 (2H, t, J = 6.2 Hz, −CH_2−COCl), 4.17 (2H, t, J = 6.3 Hz, −CH_2−O−), 4.78 (1H, quartet, CH).

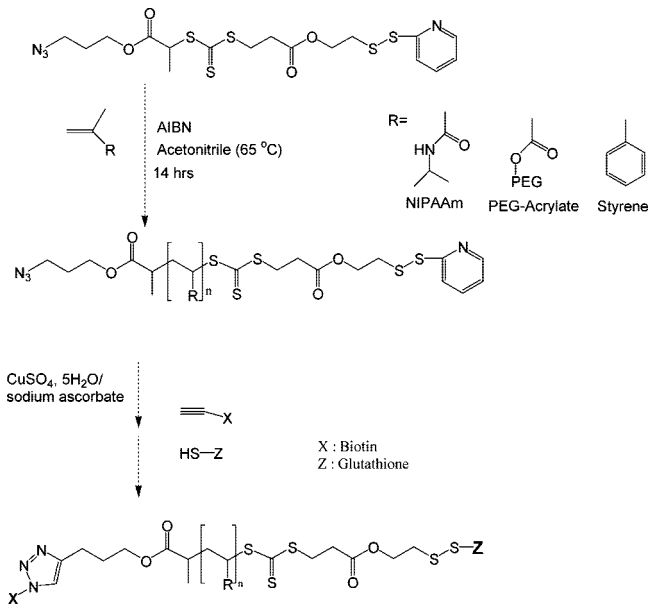
Step 5: Synthesis of 3-Azidopropyl-2-(2-(2-pyridyldisulfanyl)ethoxycarbonyl)ethyldithiocarboxysulfanyl Propionate (Product **6***).* This step required additional care to dry all the components of the reaction mixture to avoid the degradation of the RAFT agent during the reaction. 3.3 g (0.01 mol) of product **4** obtained in a previous step was dissolved in anhydrous DCM. DCM was then partially removed to dry RAFT agent by azeotropic distillation. A solution of hydroxyethyldithiopyridine (**5**, Scheme 1) (1.8 g, 0.01 mol diluted in 10 mL of dried DCM) was added at room temperature. Triethylamine solution (1.1 equiv of alcohol) was added dropwise at 0 °C for 1 h to the mixture. The precipitate formation was observed. The reaction mixture was stirred at room temperature for another 2 h. The product was then purified by extraction using distilled water and DCM (twice at pH = 2 and twice deionized water). The organic phase was dried using sodium sulfate. The solvent was removed under vacuum at room temperature. The crude product was purified by silica gel column chromatography (hexane/ethyl acetate: 60/40 vol %). After elimination of solvents under vacuum, yellow oil (product **6**, Scheme 1) was obtained (yield 80%). ^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm from TMS): 1.59 (3H, d, −CH_3), 1.94 (2H, q, J = 6.1 Hz, −CH_2−CH_2−CH_2−), 2.79 (2H, t, J = 6.1 Hz −CH_2−CH_2−COO−), 3.05 ppm (2H, p, −CH_2−S−S), 3.41 (2H, t, J = 6.6 Hz, −CH_2−N_3), 3.65 (2H, t, J = 6.2 Hz, −C−S−CH_2−CH_2), 4.0−4.40 (4H, t, J = 6.3 Hz, −CH_2−O−), 4.78 (1H, quartet, CH), 7.1 (1H, m, aromatic hydrogen meta to nitrogen, 7.65 (2H, m, para to nitrogen and para to thiol),

Table 1. Recipes of the RAFT Polymerizations Performed and the Properties of the Polymers Generated

						M_n (g/mol)			
run	monomer	T (°C)	[M] (M)	[M]/[RAFT]/[I][a]	conv[b] (%)	theor[c]	GPC[d]	NMR	PDI[e]
1	HPMA	65	0.96	100.0/1.0/0.2	90	13 400	16 200	65 000	1.35
2	MMA	65	0.92	100.0/1.0/0.2	90	9500	12400	nd	1.65
3	MMA	70	0.92	100.0/1.0/0.2	90	9500	13000	nd	1.70
4	styrene	70	0.94	103.2/1.0/0.2	78	8900	8400	8500	1.08
5	styrene	70	0.94	103.2/1.0/0.2	46	5400	5300	5400	1.09
6	styrene	70	0.94	200.0/1.0/0.2	65	14000	14000	15000	1.11
7	OEG-A	65	0.49	22.0/.0/1.0/0.2	72	7600	7500	8100	1.12
8	OEG-A	65	0.49	44.0/1.0/0.2	68	14000	12500	15300	1.15
9	NIPAAm	65	1.02	39.0/1.0/0.2	65	3400	3200	3600	1.12
10	NIPAAm	65	1.02	95.4/1.0/0.2	74	8500	8100	7400	1.14
11	NIPAAm	65	1.02	200.0/1.0/0.2	70	16300	16200	13500	1.08

[a] [M], [RAFT], and [I] are the feed concentration of momonomer, RAFT agent, and initiator, respectively. [b] Conversion is monomer conversion. All polymerizations were performed in acetonitrile. [c] Theoretical molecular weight was calculated by M_n = (conversion (%) × [M]/[RAFT] × $MW_{(monomer)}$) + MW_{RAFT}. [d] GPC molecular weights were determined by GPC (mobile phases: DMAc for HPMA, OEG-A, and NIPAAm; THF for styrene and MMA). [e] PDI is polydispersity index. All polymerizations were performed in acetonitrile.

Scheme 2. RAFT-Mediated Synthesis of Heterotelechelic Polymers and Their Use in Selective Bioconjugation Reactions



8.45 (1H, q, aromatic hydrogen ortho to nitrogen). The spectrum can be seen in Figure S1 (Supporting Information).[13]C NMR (75 MHz, $CDCl_3$): 20.14 (CH_3), 29.50 ($-CH_2-CH_2-CH_2O-$), 31.20 (CH_2-S-), 40.10 (CH_2O), 42.10 ($-O-CH_2-CH_2-SS$), 48.27 (CH_2-N_3), 62.5 ($-CH-$), 119.30 121.70, 138.02, 149.51, 159.23 (CH of Ar).171.0−175.0 ($C{=}O$), 222.10 ($C{=}S$). FT-IR (cm^{-1}): 3040, 2979, 2930, 2850, 2087, 1730, 1570, 1556, 1280, 1067, 1446, 1379, 1334, 1270, 1220, 1155, 1120, 1097, 1070, 1029, 763, 675. ESI-MS: 529.5 Da (theoretical value = 529.75 Da). This RAFT agent was found to decompose in the mass spectrometer before the ionization. Molecular peak and assigned fragments were detected, but at concentrations lower than expected.

The synthesis of hydroxyethyldithiopyridene (**5**, Scheme 1) is described in the Supporting Information (section 1.3.1).

RAFT Polymerizations. A number of polymerizations with methyl methacrylate, hydroxypropylmethacrylamide, styrene, NIPAAm, and OEG-A were performed using the new RAFT agent bearing azide and dithiopyridine functional groups. The monomer concentrations and the ratio of the monomer, RAFT, and initiator concentrations were varied throughout the polymerization experiments as tabulated in Table 1. The typical procedures are given below for polymerization of styrene, NIPAAm, and OEG-A.

In a typical polymerization of styrene, 50.0 mg (9.3×10^{-2} mmol) of the RAFT agent (bearing azide and dithiopyridine groups) and 1.0 g (9.6 mmol) of styrene were dissolved in 9 mL of acetonitrile. Following the sealing of the vial with rubber septum, the polymerization solution was purged with nitrogen for 30 min in an ice bath. A deoxygenated solution of the initiator (i.e., AIBN, 1.2 mL from 15.2 mM in acetonitrile (1.8×10^{-2} mmol)) was introduced into the reaction mixture at 0 °C using a syringe. The final concentration of the monomer, RAFT agent, and the initiator in the polymerization medium was 0.94, 9.11×10^{-3}, and 1.78×10^{-3} M, respectively ($[monomer]_0:[RAFT]_0:[initiator]_0 = 103.2: 1.0: 0.2$). The solution was further deoxygenated at 0 °C for 15 min and then placed in an oil bath at 70 °C. Aliquots (0.5 mL) were taken at predetermined time intervals and quenched via rapid cooling and exposure to oxygen. Two or three drops of hydroquinone solution (5 wt %) were added. These samples were directly analyzed by ^{1}H NMR and THF GPC to determine the monomer conversion and the molecular weight. The polymer was concentrated by partial evaporation of acetonitrile, and the polymer was precipitated in cold methanol (in an ice bath) twice to remove the nonreacted monomer and the RAFT agent. After filtration, the solid was dried in oven at 40 °C for 24 h to give a yellow-orange solid. The samples were further analyzed by MALDI-ToF and/or ESI-MS, DMAc GPC, and ^{1}H NMR.

A similar procedure was followed for polymerizations of NIPAAm and OEG-A, except the changes in the concentration of the components used. For polymerizations with NIPAAm, the final concentration of NIPAAm, RAFT agent, and the initiator was 1.02, 10.69×10^{-3}, and 2.09×10^{-3} M, respectively, yielding a ratio of $[monomer]_0:[RAFT]_0:[initiator]_0$ of 95.4:1.0:0.2. For polymerization with OEG-A, the final concentration of OEG-A, RAFT agent, and the initiator was 0.49, 22.27×10^{-3}, and 3.16×10^{-3} M, respectively, yielding a ratio of $[monomer]_0:[RAFT]_0:[initiator]_0$ of 22.0:1.0:0.2. Both NIPAAm and OEG-A polymerizations were performed at 65 °C. The samples obtained during the polymerization of NIPAAm and OEG-A were directly analyzed by ^{1}H NMR and DMAc GPC to determine the monomer conversion and the molecular weight. The polymer samples were concentrated by partial evaporation of acetonitrile, and the polymer was precipitated in cold diethyl ether (in an ice bath) two times to remove the nonreacted monomer and the RAFT agent. After filtration, the solid was dried in oven at 40 °C for 24 h to give a yellow-orange solid. The purified products were further analyzed by ^{1}H NMR, DMAc GPC, MALDI-ToF, and FT-IR.

Poly(NIPAAm) (M_n: 3200 g/mol by GPC, PDI: 1.12, conversion: 65%, run 9, Table 1) was copolymerized using OEG-A as the comonomer. In a typical copolymerization reaction, the concentration of OEG-A, macroRAFT agent, and the initiator was 0.52, 12.75×10^{-3}, and 1.02×10^{-3} M, respectively, yielding a ratio of $[monomer]_0:[macroRAFT]_0:[initiator]_0$ of 39.0:1.0:0.2. 0.405 g (1.27×10^{-4} mol) of the macroRAFT agent (i.e., poly(NIPAAm)-RAFT) was dissolved in 5 mL of acetonitrile. 2.25 g (5×10^{-3} mol) of OEG-A, 8.3 mg (1×10^{-5} mol) of AIBN, and 4.6 mL of acetonitrile were added. The solution was purged for 30 min with nitrogen to remove trace of oxygen. After polymerization, reaction mixture was concentrated by partial evaporation of acetonitrile and precipitated

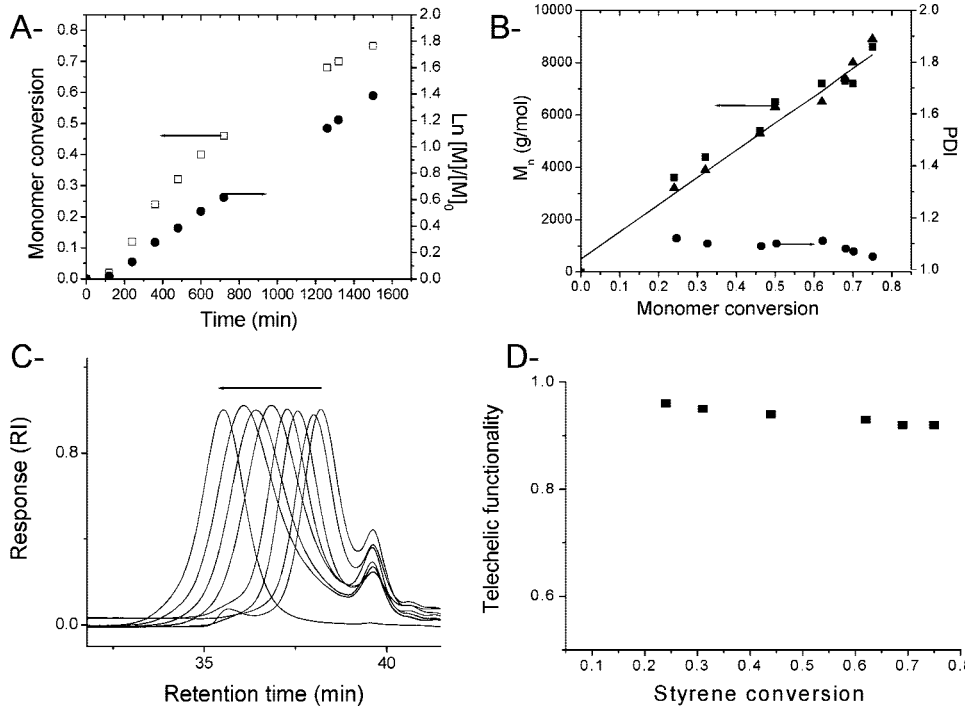


Figure 1. Results of styrene polymerization ([St]/[RAFT]/[AIBN] = 103.2:1.0:0.2, [St] = 0.94 M, [RAFT] = 9.11×10^{-3} M, [AIBN] = 1.78×10^{-3} M in acetonitrile at 75 °C). (A) Evolution of monomer conversions (□) and ln([M]/[M]$_0$) (●) with time. (B) Evolution of polydispersity index (PDI) (●) and the number-average molecular weight (M_n) determined by ^{1}H NMR after purification (■), by GPC (▲), and by theoretical calculations (—) vs monomer conversion. (C) GPC traces at varying monomer conversions (the direction of arrow shows increasing conversion). (D) Evolution of the telechelic functionality vs monomer conversion. The telechelic functionality is the mole ratio of the dithiopyridine end group to the azide end group on the polymer. It was calculated by the ratio of the integration of the peak at 8.4 ppm (representing ω-pyridine end group) to the integration of the peak at 4.0 ppm (representing α-azido end group) from ^{1}H NMR spectra.

in diethyl ether twice. The final product was dried in oven at 40 °C for 24 h to yield a yellow powder. The product was analyzed by ^{1}H NMR and DMAc GPC.

Conjugations of Biomolecules to Poly(NIPAAm). Synthesis of biotin amidopropyne, its conjugation to α-azide, ω-dithiopyridine poly(NIPAAm), conjugation of glutathione and bovine serum albumine (BSA) to α-biotin, ω-dithiopyridine poly(NIPAAm), affinity binding of avidin to α-biotin, ω-BSA poly(NIPAAm), and the characterization of bioconjugates are given in the Supporting Information (sections 1.3.2−1.3.9).

Results and Discussion

Synthesis of Heterotelechelic Polymers. A new trithiocarbonate RAFT agent bearing an azide and a dithiopyridine group at R and Z fragments, respectively, was synthesized (Scheme 1). ^{1}H NMR of the new RAFT agent showed the presence of the protons characteristic to pyridyl group at 7.1, 7.6, and 8.5 ppm and the protons characteristic to methylene group, adjacent to the azide group, at 3.4 ppm (Figures S1−S3 in the Supporting Information). Analysis by ^{13}C NMR and ESI-MS confirmed also the successful synthesis of the expected RAFT agent structure.

The RAFT agent was then tested in the polymerization of various monomers: styrene, *N*-isopropylacrylamide (NIPAAm), oligo(ethylene glycol) acrylate (OEG-A), hydroxypropyl methacrylamide (HPMA), and methyl methacrylate (MMA) (Scheme 2). A number of polymerizations were performed varying the ratio of the monomer, RAFT agent, and initiator concentration as summarized in Table 1. In the case of styrene, OEG-A, and NIPAAm polymerizations, ln [M]$_0$/[M] vs time exhibited a linear relationship until 75% monomer conversions, indicating constant propagating radical concentration throughout the polymerization (Figures 1 and 2 and Figure S4 in the Supporting Information). A short inhibition period was observed at the beginning of polymerizations which can be attributed to the presence of oxygen traces or the fragmentation of the RAFT agent. The linear evolution of the molecular weights (determined by GPC) with increasing monomer conversions and also the low PDIs (<1.2) indicated the well-controlled polymerization mechanism for conversions up to 75% (Figures 1 and 2 for styrene and NIPAAm; Figure S4 in the Supporting Information for OEG-A). The theoretical and the experimental molecular weights of the polymers (determined by GPC for poly(styrene) and by NMR for poly(NIPAAm) and poly(OEG-A)) were in good agreement for styrene, OEG-A, and NIPAAm, indicating the high efficiency of the RAFT agent (Table 1). The GPC traces (Figures 1 and 2 and Figure S4 in the Supporting Information) exhibited monomodal distribution at low monomer conversions, while at high conversions the presence of the shoulders at low retention times indicated the presence of termination reactions.[40,58]

^{1}H NMR of the purified polymers confirmed the presence of the dithiopyridine and azide groups on the polymer chains (Figure 3 for poly(NIPAAm) and Figure S5 in the Supporting Information for poly(styrene)). While the signals of dithiopyridine group of the polymers appeared at 7.1, 7.6, and 8.5 ppm, the signals of methylene groups adjacent to azide group were observed at 4.0 and 3.4 ppm for styrene and NIPAAm polymers, respectively (Figure 3 and Figure S5 in the Supporting Information). For poly(OEG-A), while the signals of the methylene groups adjacent to azide group overlapped with the polymer's signals, the broad signal at 4.75 ppm which represented the C*H* group of the polymer's backbone last repeating unit adjacent to the trithiocarbonate functionality (Figure S4 in the Supporting Information) indicated the presence of the end group arising from the RAFT agent. The molecular weights calculated from ^{1}H NMR, assuming one dithiopyridine group per polymer chain, were also in good agreement with the theoretical molecular weights (Figures 1 and 2 and Figure S4 in the Supporting Information), indicating the efficient RAFT-mediated generation

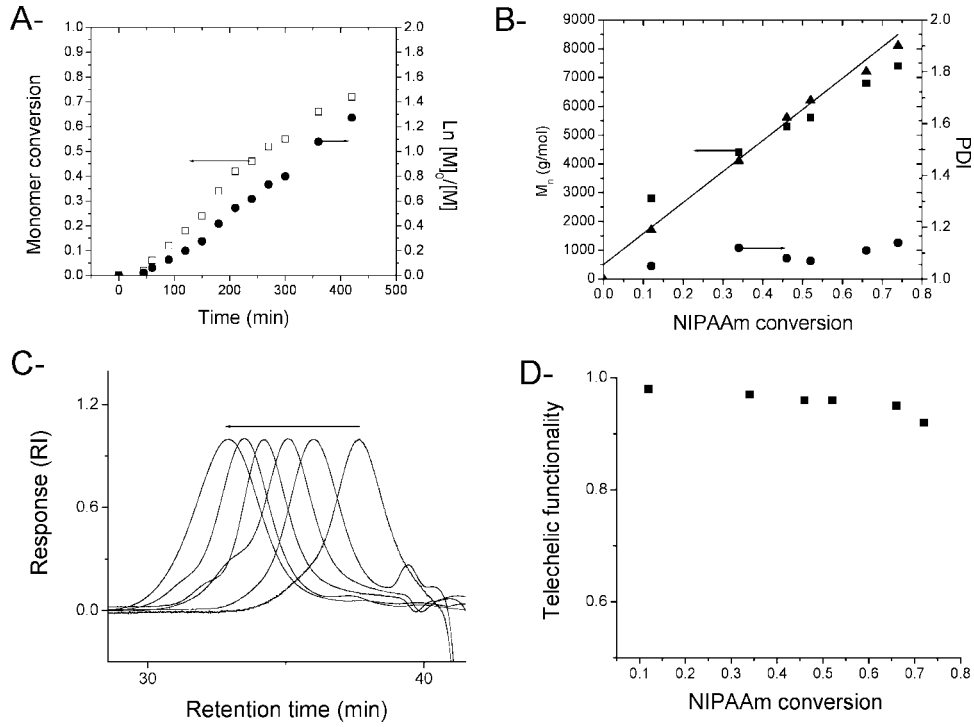


Figure 2. Results of NIPAAm polymerization ([NIPAAm]/[RAFT]/[AIBN] = 95.4: 1.0: 0.2, [NIPAAm] = 1.02 M, [RAFT] = 10.69×10^{-3} M, [AIBN] = 2.09×10^{-3} M in acetonitrile at 65 °C). (A) Evolution of monomer conversions (□) and ln([M]/[M]$_0$) (●) with time. (B) Evolution of polydispersity index (PDI) (●) and the number-average molecular weight (M_n) determined by ^{1}H NMR after purification (■), by GPC (▲), and by theoretical calculations (—) vs monomer conversion. (C) GPC traces at varying monomer conversions (the direction of arrow shows increasing conversion). (D) Evolution of the telechelic functionality vs monomer conversion. The telechelic functionality is the mole ratio of the dithiopyridine end group to the azide end group on the polymer. It was calculated by the ratio of the integration of the peak at 8.4 ppm (representing ω-pyridine end group) to the integration of the peak at 3.4 ppm (representing α-azido end group) from ^{1}H NMR spectra.

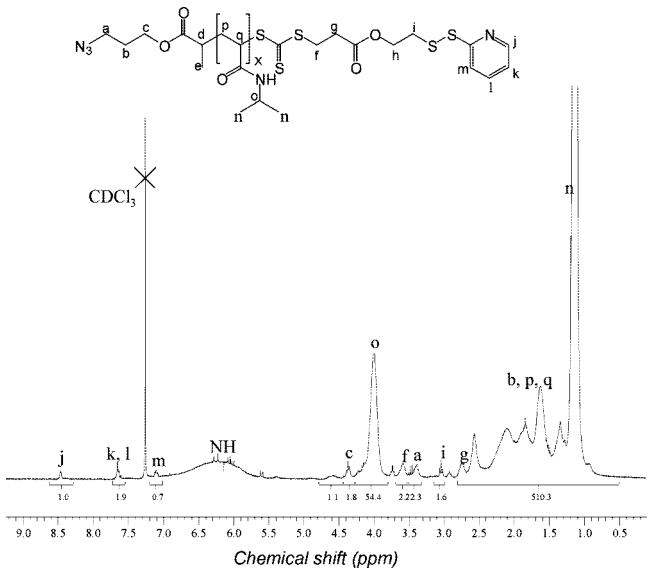


Figure 3. ^{1}H NMR spectrum of purified poly(NIPAAm) (M_n = 7400 g/mol by ^{1}H NMR and 8100 g/mol by GPC, PDI = 1.14 and conversion 74%, recorded in $CDCl_3$, 300 MHz, 300 K).

of polymers with defined end-group functionality. The telechelic functionality of styrene and NIPAAm polymers (i.e., mole ratio of the ω-dithiopyridine to the α-azide end group), calculated from ^{1}H NMR spectra, was above 0.90 for the polymers generated up to 75% monomer conversions (Figures 1 and 2). This also indicated the efficient generation of well-defined heterotelechelic polymers. While azide- and pydriyldisulfide-functionalized semitelechelic polymers were generated previously in one step using functionalized RAFT agents,[12,16,34,59–62] the direct synthesis of heterotelechelic polymers combining these two different functionalities has been described here for the first time. The heterotelechelic functionality of the polymers decreased slightly with increasing monomer conversion, which was expected for the RAFT polymerization in which the generation of the polymer products through the coupling reactions (of initiator and propagating polymeric radicals) is unavoidable.[41] The ESI-MS and MALDI-ToF analyses of poly(NIPAAm) (M_n: 3200 g/mol by SEC, PDI: 1.12, conversion: 65%; run 9, Table 1) revealed that the heterotelechelic polymer chains constituted the major population in the sample while the polymers terminated with the initiator (AIBN) fragments were also present to a less extent (Figure S6 in the Supporting Information).

Here it is worth noting that significant loss of azide moiety in polymerization of various monomers including acrylamides, acrylates, and styrene have been reported by Perrier et al. in private communications. The NMR and FTIR analyses of the poly(NIPAAm) and poly(styrene)s synthesized in different batches (at least three) under the experimental conditions used in our study consistently showed the retention of functional azide end group on the polymers with good efficiency (Figure 3 for poly(NIPAAm) and Figure S5 for poly(styrene) and Figures S14 and S15 for poly(NIPAAm) synthesized separately using a RAFT agent having azide and carboxylic acid functionality). While the presence of nonattributable populations in addition to the expected product population on the ESI-MS spectra of the polymer samples (Figure S6 in the Supporting Information) was not overlooked in our study, it is clear from differences in observations of different groups that the polymerization conditions play a vital role in retention of the azide group functionality.

The new RAFT agent was further tested in the polymerization of HPMA and MMA monomers where no control over the polymerization was observed (Figure S7 in the Supporting

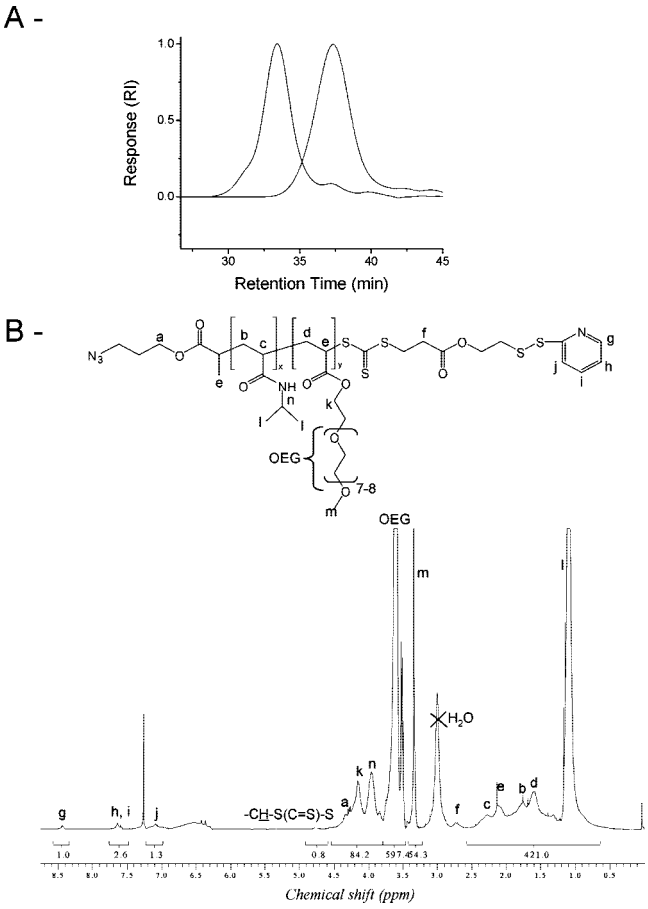


Figure 4. Chain extension of α-azide, ω-dithiopyridine poly(NIPAAm) (M_n: 3200 g/mol by GPC, PDI: 1.12, conversion: 65%, run 9, Table S1) with OEG-A. DMAc GPC traces of poly(NIPAAM) macroRAFT agent before and after the chain extension and ^{1}H NMR spectrum of purified poly(NIPAAm)-*b*-poly(OEG-A) (recorded in $CDCl_3$, 300 MHz, 300 K).

Information). High PDI values of the polymers and the nonlinear relation between the molecular weight and the monomer conversion indicated the absence of the RAFT-controlled polymerization mechanism.[63] This was attributed to the low dissociation of the RAFT agent due to its inappropriate R group for the polymerization of methacrylates and methacrylamides.[64,65]

To test the living behavior of the α-azide, ω-dithiopyridine functional telechelic polymers generated by the new RAFT agent, poly(NIPAAm) (M_n: 3200 g/mol by GPC, PDI: 1.12, conversion: 65%, run 9, Table 1) was used as a macroRAFT agent for the copolymerization of OEG-A. GPC analysis of the polymerization mixture (Figure 4) showed the clear shift of the macroRAFT agent peak to the low retention times, which proved that more than 90% of the poly(NIPAAm) chains underwent copolymerization. This confirmed that above 90% of poly(NIPAAm) chains had an active RAFT functionality. ^{1}H NMR analysis (Figure 4) of the purified poly(NIPAAm)-*b*-poly(OEG-A) showed the slight shift of the proton signal attributed to C*H*−S(C═S)− from 4.60 to 4.75 ppm after polymerization with OEG-A, indicating the presence of the RAFT-incorporated poly(OEG-A). Thus, the living character of the generated telechelic polymers was shown by efficient chain extension of poly(NIPAAm) with OEG-A.

Selective Bioconjugations to α-Azide, ω-Dithiopyridine Poly(NIPAAm). The heterotelechelic functionality of the generated polymers for selective bioconjugation reactions was tested by reacting α-azide, ω-dithiopyridine poly(NIPAAm) with model biomolecules (Scheme 2). Biotin, a widely used biomolecule in biotechnology applications,[66,67] was first conjugated to α-end of heterotelechelic poly(NIPAAm) via click reaction using a specific catalyst system. The catalyst system ($CuSO_4 \cdot 5H_2O$/sodium ascorbate or $CuSO_4 \cdot 5H_2O$/Cu)[61,68,69] used in the click reaction was carefully chosen to maintain the integrity of the trithiocarbonate end group. Before performing biotin conjugation, model click reactions were carried out in the presence of the RAFT agent and a model alkyne compound, i.e., propargyl alcohol, using different catalyst systems. Table 2 summarizes the conditions used in model click reactions. The percentage of the degradation of the RAFT agent was evaluated via the ratio of the integration of the peak at 4.80 ppm (which represents C*H* adjacent to trithiocarbonate) to the integration of the peak at 4.17 ppm (CH_2O) (Figure S1, bottom spectrum, Supporting Information). As can be seen in Table 2, degradation of the RAFT agent was observed in the presence of *N*,*N*,*N*′,*N*″,*N*″-pentamethyldiethylenetriamine (PMDETA) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). The click reaction performed with $CuSO_4 \cdot 5H_2O$/sodium ascorbate as catalyst system was also monitored via FT-IR (Figure S8, Supporting Information). While the azide group band (∼2100 cm^{-1}) disappeared almost completely, the thiocarbonate band (∼1100 cm^{-1}) was found to retain completely, indicating the high reaction yields without the degradation of the RAFT agent.

For the α-end-biofunctionalization of poly(NIPAAm) via click chemistry, an alkyne-modified biotin (i.e., biotin-amidopropyne) was prepared first (Figure S3, Supporting Information) and reacted with poly(NIPAAm) using $CuSO_4 \cdot 5H_2O$/sodium ascorbate as catalyst system. After the reaction and purification by dialysis, the polymer (M_n: 7400 g/mol by ^{1}H NMR, 8100 g/mol by GPC; PDI = 1.14, heterotelechelic functionality: 0.92) was analyzed by ^{1}H NMR and by FTIR (Figure 5). In NMR spectrum, the appearance of the proton signals at 7.75 and at 4.60 ppm, attributed to C*H* of the triazole ring and CH_2 in adjacent position to triazole ring, respectively, indicated the formation of triazole ring on the polymer upon click reaction. Biotin signals at 6.3−6.4 and 4.15−4.25 ppm confirmed the conjugation of biotin. Moreover, the absence of the azide group band (at 2100 cm^{-1}) in the FT-IR spectrum of the purified biotin-attached polymer indicated the near-stochiometric conjugation yield via click reaction, which supported the ^{1}H NMR analysis results (Figure 5). It is also important to note that the absence of alkyne band at 2200 cm^{-1} in the FT-IR spectrum of the purified biotin-attached polymer revealed that the excess of alkyne-modified biotin (nonconjugated biotin) was totally removed from the polymer product during purification, and thus the biotin signals observed in NMR spectrum of the polymer were solely due to the conjugated biotin. In addition, the signals at 7.2, 7.6, and 8.5 ppm in the NMR spectrum confirmed also that the ω-dithiopyridine end group of the polymer was not affected by the click reaction conditions. MALDI-ToF analysis of poly(NIPAAm) (M_n = 3200 g/mol by GPC, PDI = 1.12, conversion: 65%) after biotin conjugation showed the increase in the mass of the starting polymer exactly equal to the mass of the conjugated biotin−amidopropyne (+281 amu) without any indication of the cleavage of the ω-end group from the polymer (Figure S9 in the Supporting Information).

The biotinylation degree of α-biotin, ω-dithiopyridine poly(NIPAAm) after the click reaction and subsequent purification was further evaluated by HABA assay.[67,70] In this assay, the decrease in the visible light absorption of HABA/avidin complex solution ($\epsilon_{at\ 500\ nm}$ = 35 500 M^{-1} cm^{-1}) after the incremental addition of the biotin-terminated polymer solution was measured via a UV−vis spectrometer. The biotin in the solution replaces the HABA in the HABA/avidin complex because of the higher association constant of the biotin/avidin

Table 2. Stability of the RAFT Agent under Varying Click Reaction Conditions[a]

molar ratio of the reactants	solvent	T (°C)	result
$[N_3]$:[alkyne]:[CuBr]:[DBU] = 100:200:1:1	DCM	40	full degradation
$[N_3]$:[alkyne]:[CuBr]:[DBU] = 100:200:1:1	DCM	25	80% degradation
$[N_3]$:[alkyne]:[CuBr]:[PMDETA] = 100:200:1:1	DCM	25	35% degradation
$[N_3]$:[alkyne]:$[CuSO_4 \cdot 5H_2O]$:[Cu] = 100:200:1:20	water/isopropanol (1:1)	25	no degradation
$[N_3]$:[alkyne]:$[CuSO_4 \cdot 5H_2O]$:[sodium ascorbate] = 100:200:1:1	water/isopropanol (1:1)	25	no degradation

[a] ^{1}H NMR was used to determine the degradation of the RAFT agent as described in the experimental and results sections.

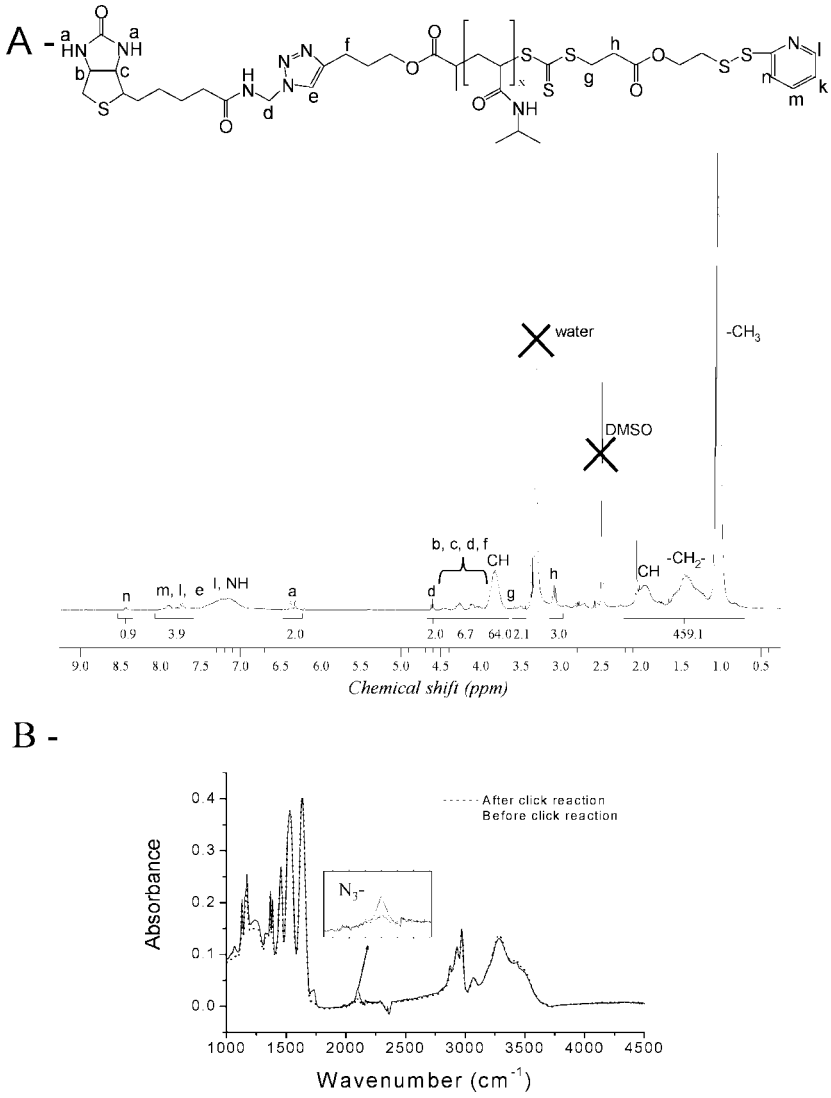


Figure 5. Characterization of α-biotin, ω-dithiopyridine poly(NIPAAm) (M_n: 7400 g/mol by ^{1}H NMR, 8100 g/mol by GPC; PDI =1.14, telechelic functionality: 0.92). (A) ^{1}H NMR spectrum of the polymer after purification (DMSO, 300 MHz, 298 K). (B) FTIR spectrum of the pure polymer before and after the click reaction.

complex (1.3×10^{15} M^{-1}) compared to that of HABA/avidin complex, which causes the decrease in the absorption of the solution. Thus, the comparison of the absorbance decrease by the addition of biotinylated poly(NIPAAm) and that of a standard biotin solution (at known concentration) indicated clearly the near-stoichiometric modification of the polymer end group with biotin (Figure 6 and Figure S10 in the Supporting Information). The biotin functionality was close to ~90% (calculated by the ratio of the slope of polymer sample to the slope of standard biotin solution). It is important to note that the assay was carried out with the excess of HABA/avidin complex compared to the biotin sample to minimize the possible steric hindrance effects. Biotinylated polymers have been reported to bind two or three biotin binding sites of avidin.[9,57]

The integrity of ω-end group of biotinylated poly(NIPAAm) was also tested by reacting the polymer with 3-mercaptopropionic acid. The release of the byproduct, i.e., pyridine-2-thione, measured by a UV−vis spectrometer at 370 nm was quantified using a calibration curve of standard pyridine-2-thione solution (Figure S11 in Supporting Information). From this test, ω-end group was found to be integral and also the heterotelechelic functionality was found to be 92%.

At the last step, α-biotin, ω-dithiopyridine poly(NIPAAm) was reacted with thiol-containing model biomolecules, i.e., a tripeptide, reduced glutathione, and also a protein, bovine serum albumin (BSA) (Scheme 3). The yield for the reactions was calculated by measuring the concentration of the reaction byproduct, i.e., pyridine-2-thione, via a UV−vis spectrometer at 343 nm (ϵ = 8080 M^{-1} cm^{-1}) and expressed based on the mol % of dithiopyridine end groups (Figure 7). The yield for the glutathione conjugation was found to be ca. 95 ± 5%. Near-stoichiometric conjugation of glutathione to the polymer

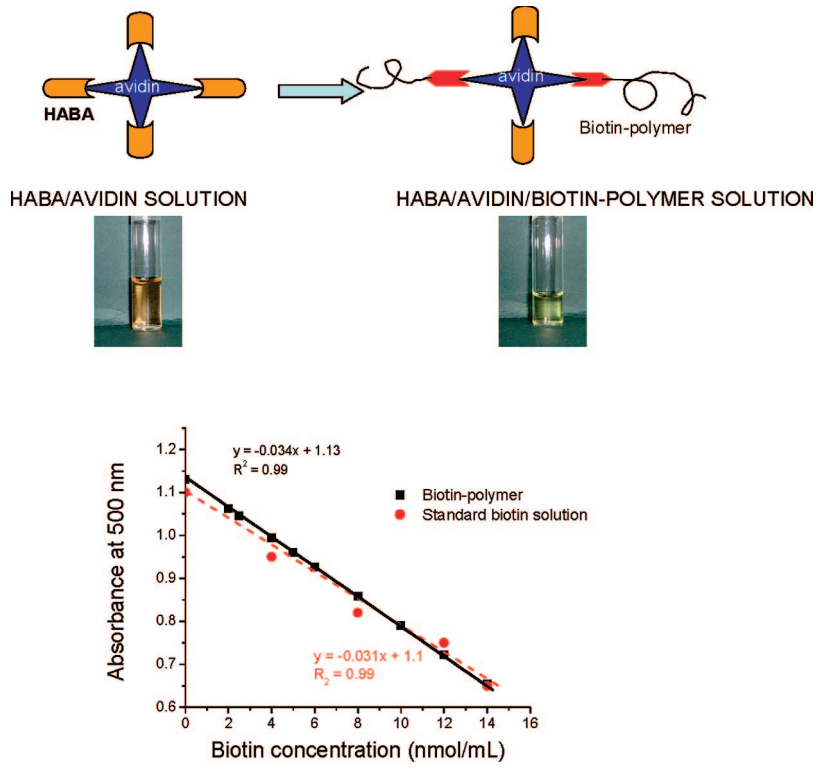
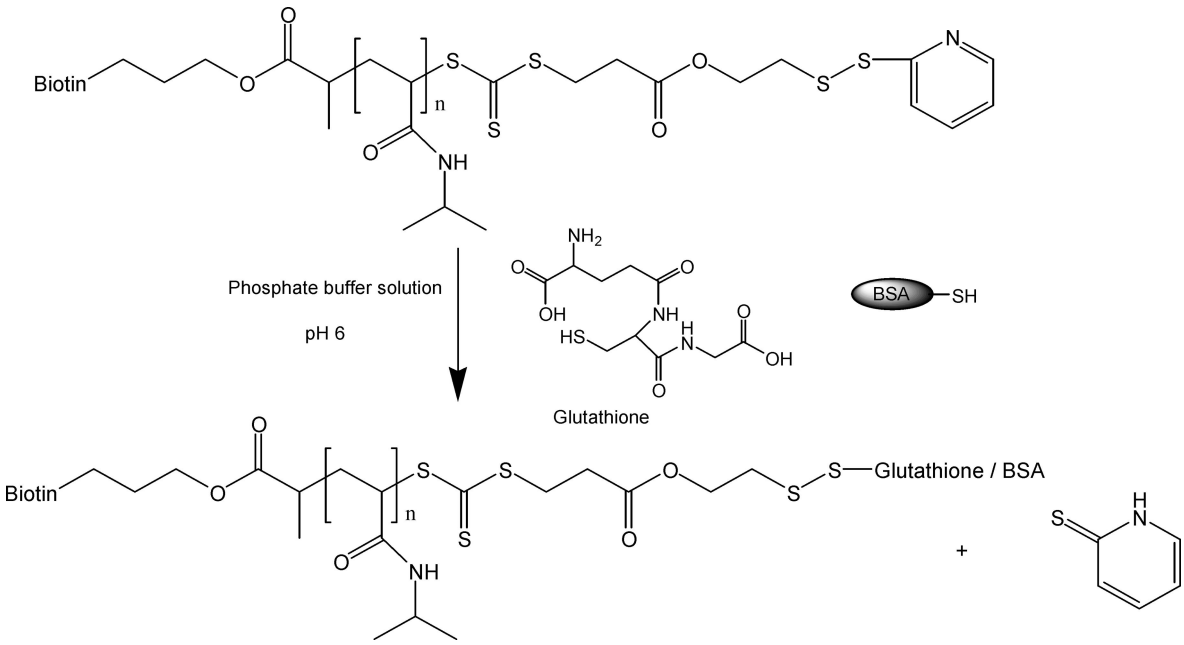


Figure 6. Schematic representation of HABA/avidin assay and the assay results of α-biotin, ω-dithiopyridine poly(NIPAAm) (M_n: 7400 g/mol by ^{1}H NMR, 8100 g/mol by GPC; PDI = 1.14, telechelic functionality: 0.92) and a standard biotin solution.

Scheme 3. Conjugation of Glutathione and BSA to α-Biotin, ω-Dithiopyridine-Functionalized Polymer



indicated the high functionality of the dithiopyridine end group. ^{1}H NMR analysis of glutathione conjugated poly(NIPAAm) after purification (Figure S12 in the Supporting Information) showed the characteristic signals of glutathione, which also indicated the conjugation of glutathione to the polymer.

In the case of BSA conjugation, when a feed molar ratio of α-biotin, ω-dithiopyridine polymer (M_n: 7400 g/mol by NMR and 8100 g/mol by GPC; PDI: 1.14, heterotelechelic functionality 0.92) to BSA of 5 was used, 95 mol % of free-thiols-bearing BSA was conjugated with a polymer chain (Figure S13 in the Supporting Information) as quantified by Ellman's assay[72,73] determining the free thiol content of BSA before and after the conjugation with polymer. This result was also verified by the UV−vis analysis of the conjugation reaction mixture (Figure 7), allowing determination of the concentration of pyridine-2-thione released upon conjugation. Both Ellman's assay and UV−vis analysis results indicated that ~10% of the polymer chains in the reaction mixture could be modified with BSA. The relatively lower reactivity of polymer's end group in BSA conjugation was attributed to the poor accessibility of the BSA's nonoxidized cysteine residue to the end group of the polymer due to the steric hindrance between two macromolecules, i.e.,

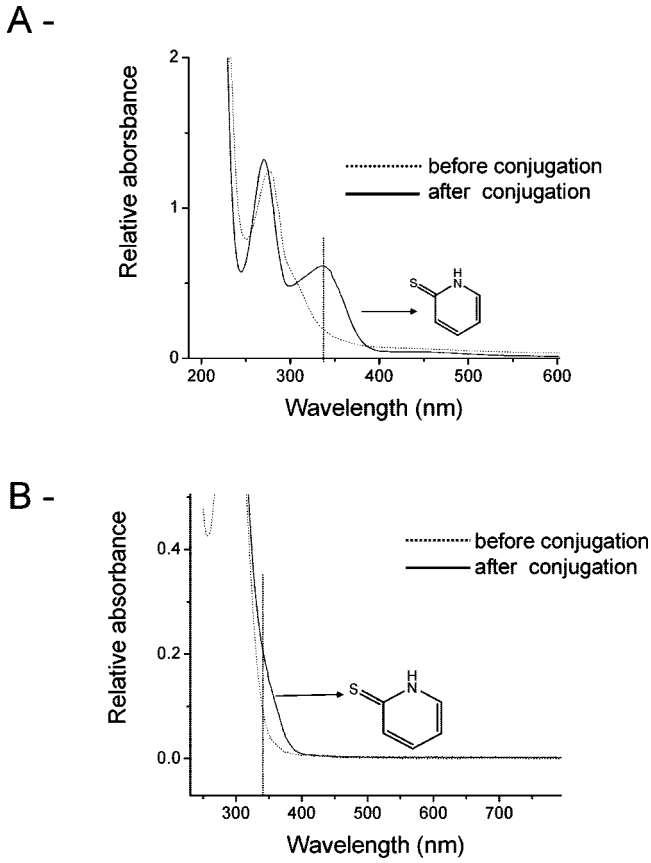


Figure 7. Glutathione and BSA conjugation to α-biotin, ω-dithiopyridine poly(NIPAAm) (M_n: 7400 g/mol by NMR and 8100 g/mol by GPC; PDI: 1.14, telechelic functionality 0.92). UV absorption of polymer solution (in phosphate buffer) before and after conjugation. Conjugation yield was calculated by the following equation: yield (%) = [(absorbance/8080)/$C_{polymer}$] × 100, in which $C_{polymer}$ is the polymer molar concentration calculated based on the molecular weight obtained by NMR.

protein and polymer. While the conjugation reactions were performed at pH 6.0 considering relatively rapid hydrolysis of the RAFT agents at basic pHs[71] and high reactivity of pyridyldisulfide groups toward thiols over a wide pH range,[43] insufficient ionization of the free thiol on BSA at slightly acidic reaction pH might be another factor contributing to relatively less efficient reaction between polymer and BSA.

The conjugation of polymer to BSA was evidenced by the appearance of a lower retention time trace on GPC chromatogram (Figure 8). Parts A and B of Figure 8 show the GPC traces of BSA before and after conjugation, respectively. The presence of a lower retention time trace after conjugation reaction confirmed the formation of BSA polymer conjugate. The GPC trace at higher retention time (same as BSA before conjugation) indicated the presence of nonreacted BSA. Here, it should be noted that 55 mol % of total BSA is not available for conjugation[15] due to the lack of free thiol on cysteine-34 residue as a result of intermolecular oxidation and/or thiol−disulfide exchange reactions in lyophilized form of commercially available BSA. While the presence of BSA dimers and unimers could be observed clearly via polyacrylamide gel electrophoresis during our experiments (data not shown), the aqueous GPC measurements did not show separate traces for dimers and unimers of BSA as seen in Figure 8A. This was attributed to the lack of resolution for protein samples. After further incubation with biotin's affinity protein,[57] i.e. avidin, the GPC trace of α-biotin, ω-BSA poly(NIPAAm) shifted to a lower retention time, which was attributed to the formation of the

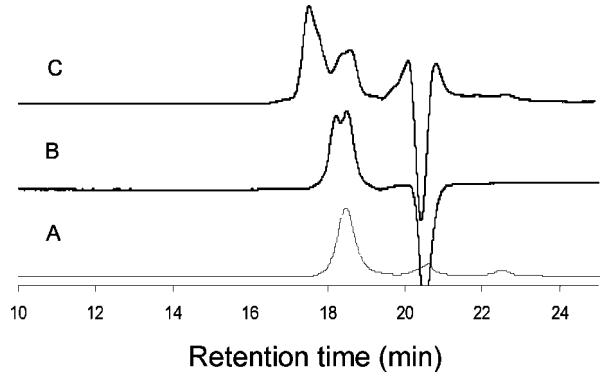


Figure 8. GPC chromatograms of BSA (A), BSA after conjugation with α-biotin, ω-dithiopyridine poly(NIPAAm) (M_n: 7400 g/mol by ^{1}H NMR, 8100 g/mol by GPC, PDI: 1.14, telechelic functionality: 0.92) (B), and the latter after incubation with avidin (C).

affinity complex of the polymer with avidin via its α-biotin end. The combination of the GPC results suggested that the ω-dithiopyridine and α-biotin functionality of poly(NIPAAm) could be used efficiently for covalent and affinity conjugation to relevant proteins.

Conclusion

In this report, we have shown the utility of the RAFT technique in the direct synthesis of α,ω-heterotelechelic polymers ready for selective bioconjugations. A new RAFT agent bearing an azide and a dithiopyridine group at its R and Z fragments was used to generate well-defined heterotelechelic polymers in one step. The controlled polymerization mechanism was obtained for polymerization of styrene, NIPAAm, and OEG-A using the new RAFT agent. Heterotelechelic functionality of the polymers was proven by the successful conjugation of poly(NIPAAm) with alkyne-modified biotin and thiol-bearing peptide/protein (i.e., glutathione and BSA) via selective chemistries. Heterobiofunctionalized poly(NIPAAM), i.e., α-biotin, ω-BSA poly(NIPAAM), was further modified via bioaffinity interactions using biotin's affinity protein, i.e., avidin.

We believe that the direct synthesis of well-defined heterotelechelic polymers having functional groups allowing the chemoselective bioconjugations is significant for enhancing the versatility of polymers for bioconjugations and biorelated applications. Our further efforts would be toward the direct generation of the well-defined heterobiofunctional polymers using the RAFT technique and also the applications of the RAFT-generated, well-defined heterotelechelic polymers in biomedical field.

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Supporting Information Available: Experimental methods, characterization of the RAFT agent, polymers and conjugation reactions, and Figures S1−S13. This material is available free of charge via the Internet at http://pubs.acs.org.

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