Efficient Synthesis of Linear Multifunctional Poly(ethylene glycol) by Copper(I)-Catalyzed Huisgen 1,3-Dipolar Cycloaddition

Xin-Ming Liu, Ashish Thakur, and Dong Wang*

Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, 986025 Nebraska Medical Center, COP 3026, Omaha, Nebraska 68198-6025

Received April 20, 2007; Revised Manuscript Received June 28, 2007

Poly(ethylene glycol) (PEG) is a versatile biocompatible polymer. Improvement of its limited functionality (two chain termini) may significantly expand its current applications. In this communication, a simple and yet highly efficient strategy for the synthesis of linear multifunctional PEGs with "click" chemistry is reported. A short acetylene-terminated PEG was linked by 2,2-bis(azidomethyl)propane-1,3-diol using Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition in water at room temperature. High-molecular-weight PEGs with pendant hydroxyl groups were obtained and characterized by ^{1}H NMR and size-exclusion chromatography. A prototype bone-targeting polymeric drug delivery system was also successfully synthesized based on this new method. It demonstrates strong biomineral-binding ability and the ease of incorporating therapeutic agents into the delivery system. This simple "click" reaction approach provides a useful tool for the development of novel functional polymers and their conjugates for biomedical applications.

Introduction

Poly(ethylene glycol), or PEG, is a water-soluble polymer that has been widely used in biomedical applications. It is known to be nonimmunogenic and has superior biocompatibility.[1,2] Several PEG conjugated (PEGylated) therapeutic agents have been approved by the U. S. Food and Drug Administration for various clinical applications.[3−5] Different from other water-soluble biocompatible polymers, such as *N*-(2-hydroxypropyl)-methacryl amide (HPMA) copolymer[6] and polyglutamic acid (PGA),[7] the functionality of PEG is limited to its two chain termini regardless of the molecular weight. To overcome this limitation, approaches have been made to synthesize linear multifunctional PEGs.[8−11] The methods that have been developed so far involve multiple reaction steps. The yields and molecular weights of the resulting products are moderate. As a novel and simple approach, we here report the synthesis of linear multifunctional PEGs with a "click" reaction.

"Click" chemistry is a newly identified classification for a set of powerful and selective reactions that require only benign reaction conditions with simple work-up and purification procedures and yet can rapidly create molecular diversity through the use of reactive modular building blocks.[12−15] The Cu(I)-catalyzed variant of the Huisgen 1,3-dipolar cycloaddition of azides and alkynes to form 1,2,3-triazoles has emerged as the most reported "click" reaction. It is characterized by high reaction yields, mild reaction conditions, tolerance of water, simple workup, good functional group compatibility, and strong reliability.[14,15] When 2,2-bis(azidomethyl)propane-1,3-diol was used as a difunctional azide reactant, a very high reaction rate was observed potentially due to a self-catalyzing mechanism.[16] Practically, it is easy to introduce azides and acetylenes into organic compounds, and these structures are stable under other reaction conditions. These unique characteristics have made the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition a powerful linking reaction in drug discovery,[17−19] polymer synthesis,[20−23] and nanoparticle[24] and biomacromolecule functionalization.[25,26]

In this study, acetylene-terminated PEG segments (derivatized from PEG 2000 diol) were joined by 2,2-bis(azidomethyl)-propane-1,3-diol via the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition to yield the linear multifunctional PEGs. A prototype bone-targeting polymeric drug delivery system was also successfully synthesized using this new method.

Experimental Section

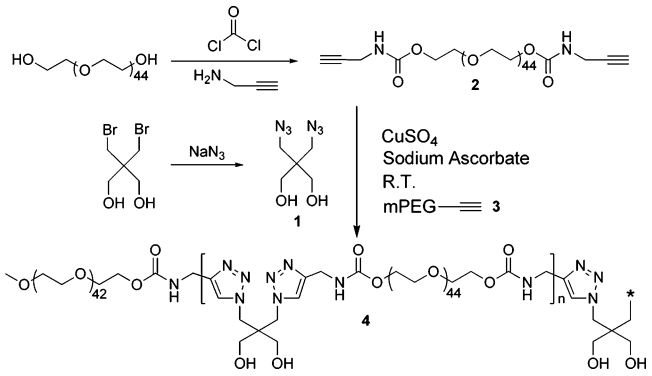
Materials and Methods. Polyethylene glycol (PEG, $M_w = 2000$) was purchased from Sigma (St. Louis, MO). Polyethylene glycol monomethylether (mPEG, $M_w = 1900$) was purchased form Alfa Aesar (Ward Hill, MA). Alendronate (ALN) was purchased from Ultratech India Ltd. (New Mumbai, India). 2,2-Bis-(bromomethyl)propane-1,3-diol and phosgene toluene solution (20%) were purchased from Aldrich (Milwaukee, WI). LH-20 and Sephadex G-25 resins were obtained from GE HealthCare (Piscataway, NJ). Hydroxyapatite (HA, DNA-grade) was purchased from Bio-Rad Laboratories (Hercules, CA). Propargyl amine, tripropargylamine, 3-bromo-1-propanol, sodium azide, sodium ascorbic acid, $Cu(MeCN)_4PF_6$, and copper sulfate were purchased from Acros (Morris Plains, NJ). All solvents and other reagents if not specified were purchased from Fisher Scientific (Pittsburgh, PA) or Acros.

^{1}H and ^{13}C NMR spectra were recorded on a 500 MHz NMR spectrometer (Varian, Palo Alto, CA). The weight average molecular weight (M_w) and number average molecular weight (M_n) of copolymers were determined by size-exclusion chromatography (SEC) using the ÄKTA FPLC system (GE HealthCare) equipped with UV and refractive index (Knauer, Berlin, Germany) detectors. SEC measurements were performed on Superose 6 (10/300 GL) or Superdex 200 (10/300 GL) columns with phosphate-buffered saline (PBS; pH = 7.3) as the eluent. PEG or poly(ethylene oxide) (PEO) standards with narrow polydispersities were used as calibration standards. The UV/vis spectra were recorded on a UV/vis spectrophotometer (UV-1601PC, Shimadzu, Kyoto, Japan).

* Author to whom correspondence should be addressed. Phone: (402) 559-1995. Fax: (402) 559-9543. E-mail: dwang@unmc.edu.

10.1021/bm070430i CCC: $37.00
Published on Web 08/10/2007

Scheme 1. Synthesis of Linear Multifunctional PEG via Cu(I)-Catalyzed Huisgen 1,3-Dipolar Cycloaddition



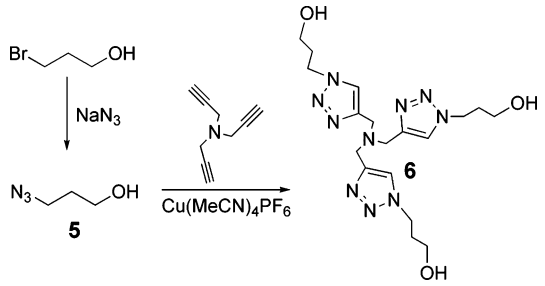
Synthesis of 2,2-Bis(azidomethyl)propane-1,3-diol (1). 2,2-Bis-(bromomethyl)propane-1,3-diol (4 g, 15 mmol, recrystallized from toluene and water) was dissolved in dimethylformamide (DMF, 30 mL). NaN_3 (4 g, 62 mmol) was then suspended in this solution. This mixture was stirred at 120 °C overnight and filtered to remove NaN_3 and NaBr. After the removal of DMF, dichloromethane (DCM, 20 mL) was added to the residue. The resulting precipitate was filtered off, and the filtrate was evaporated to dryness. The residue was subjected to a standard diethyl ether/aqueous NaCl extraction. The organic phase was dried with Na_2SO_4 and evaporated to dryness. The crude product was further purified by silica column chromatography (chloroform/methanol = 20:1). Yield: 75.2%. ^{1}H NMR ($CDCl_3$) δ (ppm): 3.61 (s, 4H), 3.41 (s, 4H), 2.75 (br, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 63.6, 51.7, 44.8.

Synthesis of Acetylene-Terminated PEG 2000 (Acetylene PEG, 2). PEG diol 2000 (10 g, [−OH] = 10 mmol) was dissolved in dry toluene, refluxed, and dried in a vacuum to remove water. Phosgene solution (15 mL, 20% in toluene) was then added into the dried PEG with stirring. The reaction was allowed to proceed overnight in a fume hood. The excess phosgene was removed in vacuum. DCM (20 mL) was used to dissolve the viscous residue. Propargyl amine (1.65 g, 30 mmol) was then added into the solution. The reaction was allowed to proceed for 7−8 h at room temperature. The product was precipitated into diethyl ether three times and purified by a LH-20 column. Yield: 83.3%. ^{1}H NMR (D_2O) δ (ppm): 4.23 (t, PEG, $-CH_2-$), 3.89 (s, propargyl amide, $-CH_2-$), 3.68 (m, PEG, $-CH_2-$). ^{13}C NMR ($CDCl_3$) δ (ppm): 155.80, 79.74, 71.24, 70.32, 69.22, 64.01, 30.44. To confirm the 100% derivatization of PEG diol into acetylene-terminated PEG, the product was also analyzed by ^{1}H NMR ($CDCl_3$). No −OH signal (δ = 2.63 ppm) was detected.

Synthesis of Acetylene-Terminated Monomethylether PEG 1900 (Acetylene mPEG, 3). The procedure was the same as that for the synthesis of acetylene-terminated PEG 2000. Yield: 89.1%. ^{1}H NMR ($CDCl_3$) δ (ppm): 5.45 (s, propargyl amide, −NH−), 4.24 (t, mPEG, $-CH_2-$), 3.96 (s, propargyl amide, $-CH_2-$), 3.64 (m, mPEG, $-CH_2-$), 3.38 (s, mPEG, CH_3-), 2.27 (s, propargyl amide, −CH). ^{13}C NMR ($CDCl_3$) δ (ppm): 155.78, 79.74, 71.67, 71.21, 70.31, 69.20, 63.98, 58.76, 30.43.

"Click" Copolymerization of Acetylene PEG 2000 and 2,2-Bis-(azidomethyl)-propane-1,3-diol in the Presence of Acetylene mPEG (Click PEG, 4). Acetylene-terminated PEG 2000 (194.4 mg, 90 μmol), 2,2-bis(azidomethyl)propane-1,3-diol (18.6 mg, 100 μmol), acetylene-terminated mPEG (39.6 mg, 20 μmol, chain terminator), tris-(hydroxypropyltriazolylmethyl)amine (THPTA; 4.32 mg, 10 μmol stabilizing agent, see below for its synthetic procedure), and $CuSO_4{\cdot}5H_2O$ (250 μg, 1 μmol) were dissolved in 8 mL of H_2O with stirring. Sodium ascorbic acid (2 mg, 10 μmol) in H_2O (2 mL) was then added dropwise to this solution. The reaction solution was stirred at room temperature (21 °C) overnight. After the reaction, EDTA was added to the polymer solution, and it was dialyzed against H_2O for 2 days to remove Cu ions and unreacted PEG 2000. The molecular weight cutoff size of the dialysis tubing was 12 kDa for a globular protein. After dialysis, the purified polymer product was lyophilized. Yield: 76%. The purified polymer was analyzed by SEC and ^{1}H NMR. M_w = 23.5 kDa, PDI = 2.8. ^{1}H NMR (D_2O), δ (ppm): 7.97 (s, triazole, −CH), 4.48 (s, triazole−C*H*$_2$−amide, $-CH_2-$), 4.39 (s, 2,2-bis(triazomethyl)propane-1,3-diol, $-CH_2-$), 4.21 (t, PEG, $-CH_2-$), 3.68 (m, PEG, $-CH_2-$), 3.36 (s, mPEG, CH_3-), 3.34 (s, 2,2-bis(triazomethyl)propane-1,3-diol, $-CH_2-$). ^{13}C NMR ($CDCl_3$) δ (ppm): 156.33, 144.95, 124.55, 71.82, 70.44, 69.38, 64.01, 60.45, 58.91, 48.78, 46.64, 36.48.

Scheme 2. Synthesis of Tris-(hydroxypropyltriazolylmethyl)amine (THPTA)



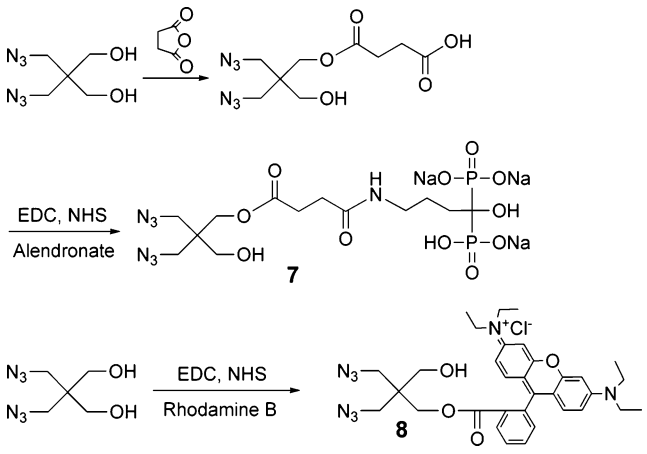
Synthesis of 3-Azido-1-propanol (5). Sodium azide (3.58 g, 55.0 mmol) was added to a solution of 3-bromo-1-propanol (1.54 g, 11.1 mmol) in DMF (9 mL). After stirring at boiling under reflux for 36 h, the mixture was filtered and coconcentrated with toluene. The residue was diluted with CH_2Cl_2 and washed twice with 10% (w/v) aqueous NaCl. The organic layer was dried, filtered, and concentrated to give the product. Yield: 71%. ^{1}H NMR ($CDCl_3$) δ (ppm): 3.73 (t, 2H, J = 6.34 Hz), 3.45 (t, 2H, J = 6.83 Hz), 2.55 (br, 1H), 1.83 (m, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 59.4, 48.3, 36.4.

Synthesis of THPTA (6).[27] Tripropargylamine (0.13 g, 1 mmol) in an acetonitrile/methanol solution (3 mL) was treated sequentially with 3-azido-1-propanol (0.35 g, 3.5 mmol), 2,6-lutidine (0.11 g, 1 mmol), and $Cu(MeCN)_4PF_6$ (2 mol % with respect to total alkyne units). Upon addition of the copper salt, the reaction was cooled in an ice bath. After the mixture was stirred at room temperature for 3 days, the reaction mixture was evaporated and dissolved in methanol. The crude product was precipitated in acetonitrile. Yield: 76%. ^{1}H NMR (DMSO-*d*) δ (ppm): 8.03 (s, 3H), 4.66 (t, 3H, J = 4.88 Hz), 4.41 (t, 6H, J = 6.83 Hz), 3.62 (s, 6H), 3.40 (q, 6H, J_1 = 2.68 Hz, J_2 = 8.54 Hz), 1.97 (m, 6H). ^{13}C NMR (DMSO-*d*) δ (ppm): 143.57, 124.16, 57.66, 47.26, 46.77, 33.17.

Synthesis of Alendronate Monomer for "Click" Copolymerization (ALN-Azide, 7). 2,2-Bis(azidomethyl)propane-1,3-diol (558 mg, 3 mmol) in 30 mL of DCM was reacted with succinic anhydride (100 mg, 1 mmol). After the disappearance of succinic anhydride, the reaction solution was evaporated. The residue was dissolved in 5 mL of water, and then 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (211 mg, 1.1 mmol) was added, followed by *N*-hydroxysuccinimide (NHS) (127 mg, 1.1 mmol). The reaction mixture was stirred for 0.5 h at room temperature. Alendronate (163 mg, 0.5 mmol) in water (pH 8) was then added dropwise. The reaction was stirred at room temperature overnight. It was precipitated in ethanol three times to obtain the final product. Yield: 67%. ^{1}H NMR (D_2O) δ (ppm): 4.04 (s, 2H), 3.51 (s, 2H), 3.42 (s, 4H), 3.19 (m, 2H), 2.70 (t, 2H, J = 6.34 Hz), 2.57 (t, 2H, J = 6.34 Hz), 1.92−1.78 (m, 4H). ^{13}C NMR (D_2O) δ (ppm): 175.49, 175.15, 74.66, 64.37, 61.17, 51.81, 44.81, 41.01, 32.09, 30.87, 24.30, 17.61.

Synthesis of Rhodamine B Monomer for "Click" Copolymerization (RB-Azide, 8). To a DCM solution of rhodamine B (479 mg, 1 mmol) was added EDC (307 mg, 1.6 mmol) followed by NHS (127 mg, 1.1 mmol). The reaction mixture was stirred for 0.5 h at room temperature. 2,2-Bis(azidomethyl)propane-1,3-diol (372 mg, 2 mmol) and 4-dimethylaminopyridine (DMAP) (13 mg, 0.1 mmol) in DCM were added dropwise. The reaction was stirred at room temperature for 8 h. The product was first purified by precipitation in ether, then by flash column chromatography (methanol/ethyl aectate = 2:10, v/v). Yield: 53%. ^{1}H NMR ($CDCl_3$) δ (ppm): 8.32 (d, 1H, J = 2.68 Hz),

Scheme 3. Syntheses of ALN-Azide and RB-Azide



7.77 (m, 2H), 7.28 (m, 1H), 7.14 (d, 2H, $J = 9.76$ Hz), 6.98 (dd, 2H, $J_1 = 9.76$ Hz, $J_2 = 1.95$ Hz), 6.81 (d, 2H, $J = 1.95$ Hz), 4.06 (s, 2H), 3.64 (q, 8H, $J = 6.83$ Hz), 3.38 (s, 2H), 3.30 (s, 4H), 1.97 (s, 1H), 1.33 (t, 12H, $J = 6.83$ Hz). ^{13}C NMR ($CDCl_3$) δ (ppm): 165.05, 158.44, 157.73, 155.56, 133.14, 132.75, 131.55, 131.33, 130.53, 130.33, 129.99, 114.55, 113.43, 96.26, 65.09, 60.65, 51.95, 46.10, 44.30, 12.58.

Synthesis of Polymeric Bone-Targeting Drug Delivery System (PEG-ALN-RB). Acetylene-terminated PEG 2000 (97.2 mg, 45 μmol), 2,2-bis(azidomethyl)propane-1,3-diol (7.44 mg, 40 μmol), ALN-Azide (2.91 mg, 5 μmol), RB-Azide (3.23 mg, 5 μmol), acetylene mPEG (19.8 mg, 10 μmol), THPTA (2.16 mg, 5 μmol), and $CuSO_4$·$5H_2O$ (125 μg, 0.5 μmol) were dissolved in H_2O (4 mL) with stirring; sodium ascorbic acid (1 mg, 50 μmol in 1 mL of H_2O) was then added dropwise to this solution. The reaction mixture was allowed to stir at room temperature for 2 days, purified by dialysis and a Sephadex G 25 column, and then freeze-dried to obtain the final product. Yield: 78%.

Alendronate and RB Content in PEG-ALN-RB. The formation of a chromophoric complex between alendronate and Fe(III) ions in acidic media was applied to determine the alendronate content in the copolymers.[28] Solutions of ferric chloride hexahydrate (4 mM) and alendronate (as a standard, 3 mM) in perchloric acid (0.2 M) were freshly prepared. The standard or sample solutions of alendronate were mixed with ferric chloride solution, and the absorbance of the complex was measured at 300 nm immediately after mixing. (The final concentrations for ferric chloride and alendronate were 0.4 and 0−0.3 mM, respectively.) All measurements were performed at room temperature against a reagent blank (0.2 M perchloric acid).

The RB content in the polymeric bone-targeting drug delivery system was determined at 565 nm by UV/vis spectrophotometer using the ethyl ester of RB as the standard.

Biomineral-Binding Ability of PEG-ALN-RB. PEG-ALN-RB (20 mg) was dissolved in PBS (10 mL). HA (100 mg) was then added to 1 mL of the solution. The mixtures were allowed to be gently agitated for 1, 5, 10, and 30 min at room temperature. HA was removed by centrifugation (10 000 rpm, 0.5 min). The UV absorbance of the supernatant was recorded and compared with that of the initial PEG-ALN-RB solution. Click PEG containing RB but no alendronate (PEG-RB) and RB were used as controls in this experiment.

Results and Discussion

Synthesis and Characterization. To achieve a simple and highly efficient synthesis of linear multifunctional PEG, the synthetic strategy was designed as shown in Scheme 1. PEG diol ($M_w = 2000$) was first modified with propargyl amine. The acetylene-terminated PEG was then connected by 2,2-bis-(azidomethyl)propane-1,3-diol with Cu(I) as the catalyst. Due to the self-catalyzing reaction that has been observed in "click" reactions using 2,2-bis(azidomethyl)propane-1,3-diol,[16] this

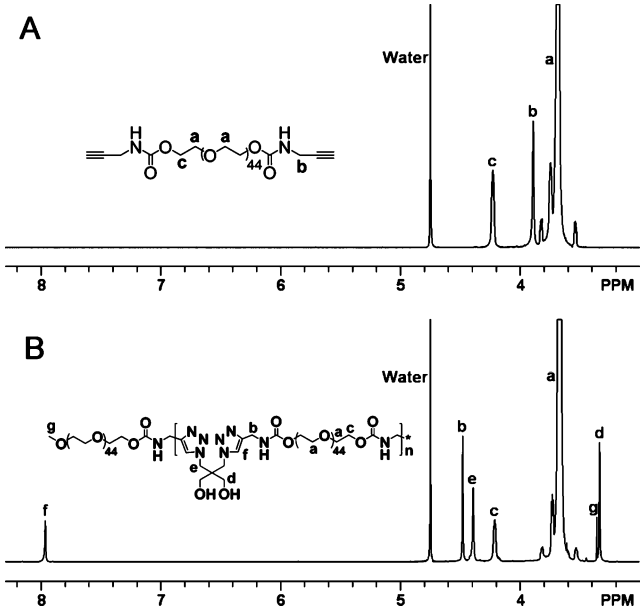


Figure 1. ^{1}H NMR spectra (D_2O) of (A) acetylene PEG 2000 and (B) a linear multifunctional PEG obtained via a "click" reaction (after dialysis) with acetylene mPEG as the chain terminator. Peak g is absent when propargyl amine is used as the chain terminator.

"click" copolymerization was expected to be very efficient. Consequently, the two hydroxyl groups of 2,2-bis(azidomethyl)-propane-1,3-diol would introduce pendant functionality to the resulting linear PEG.

One critical step in the preparation of linear, multifunctional PEG is to have 100% conversion of the two hydroxyl termini into acetylene (Scheme 1). PEG with a mono-acetylene function would inevitably act as a polymer chain terminator and leads to low-molecular-weight product. To activate the hydroxyl groups in PEG diol 2000, the dried PEG was first treated with phosgene (20% toluene solution). After removal of excess phosgene, propargyl amine was introduced. Acetylene-terminated PEG 2000 was then obtained via precipitation with ether following the elimination of propargyl amine hydrochloride salt. To completely remove residual propargyl amine, the PEG product was further purified by dialysis and a LH-20 column. The structure of the modified PEG was confirmed by ^{1}H NMR analyses as shown in Figure 1A. SEC analysis of acetylene-terminated PEG indicated that its M_w is 2.0 kDa with a polydispersity of 1.03. A tiny peak with a slightly shorter retention time appears before the main acetylene-terminated PEG peak, which may be attributed to acetylene-terminated PEG dimer that was joined in phosgene treatment. Different methods (e.g., column fractionation) are being investigated to further improve the purity of acetylene-terminated PEG 2000.

The commercially available 2,2-bis-(bromomethyl)propane-1,3-diol may contain tribromide and tetrabromide. Therefore, triazide and tetraazide could be generated in the synthesis of 2,2-bis(azidomethyl)propane-1,3-diol. In the "click" polymerization, such tri- and tetrafunctional linkers would lead to the formation of a cross-linked polymer network instead of a linear polymer. To avoid this, 2,2-bis-(bromomethyl)propane-1,3-diol was purified by repeated recrystallization in toluene and water. Its purity was confirmed by gas chromatography/mass spectrometry (GC/MS). Azidation of 2,2-bis-(bromomethyl)propane-1,3-diol was then carried out in DMF with sodium azide (Scheme 1).

The initial "click" copolymerization of acetylene-terminated PEG 2000 (10 mM) with 2,2-bis(azidomethyl)propane-1,3-diol

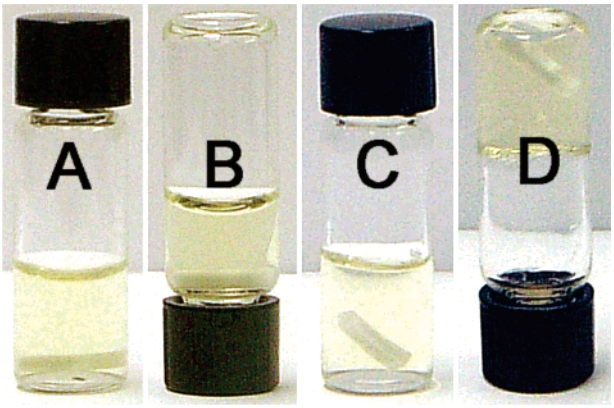


Figure 2. Without the chain terminator, the highly efficient "click" polymerization of short PEG segments leading to quick gelation. Vials A and B (inverted) show the "click" copolymerization solution at 0 h. Vials C and D (inverted) show the reaction after 1 h when gelation happens.

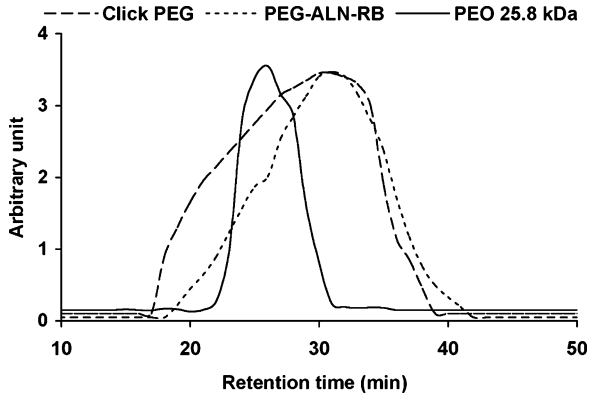


Figure 3. SEC analyses of "click" copolymerization products. A Superdex 200 column (10/300 GL) was used with PBS (pH = 7.3) as the eluent. Polyethylene oxide standard (PEO, M_w = 25.8 kDa, PDI = 1.17) is shown as a reference. Click PEG: M_w = 23.7 kDa, PDI = 2.81. PEG-ALN-RB: M_w = 14.0 kDa, PDI = 2.44.

(10 mM) was performed in H_2O at room temperature as the reaction is particularly efficient in water.[14,15] $CuSO_4 \cdot 5H_2O$ and sodium ascorbate (1.25 mM each) were used for in situ generation of the active Cu(I) as a catalyst.[16] The polymerization ended with gelation within 1 h (Figure 2). When the catalyst concentration was further reduced to 0.1 mM, gelation occurred overnight.

There are two possible explanations for the observed gelation in the "click" copolymerization. (1) Because the "click" reaction involves 2,2-bis(azidomethyl)propane-1,3-diol, which has a self-catalyzing effect,[16] the polymerization could be highly efficient in forming high-molecular-weight PEG, which leads to gelation. (2) Since triazole is a good electron donor, the newly formed triazole pair may interact with Cu(I) and form physical cross-links during the polymerization process. To explore the potential of the second possibility, the gel was washed extensively with EDTA solution (100 mM) with no gel dissolution observed over 24 h. It rules out the possibility of a Cu(I) cross-linked polymer network. Therefore, we believed that the quick gelation observed in the "click" copolymerization may be explained by the highly efficient reaction and the formation of a very high-molecular-weight PEG. To control the molecular weight and avoid gelation, propargyl amine (acetylene-terminated PEG/propargyl amine = 9.5:1) was added to the reaction as a chain terminator.[29] A polymer solution was obtained under these conditions.

^{1}H NMR analysis of click PEG shows the triazole proton at 7.97 ppm (peak f in Figure 1B) and the methylene protons from the pendant diol structure at 3.34 and 4.39 ppm (peaks d and e in Figure 1B). In addition, $-CH_2-$ adjacent to the carbamate structure at 3.89 ppm (peak b in Figure 1A) shifts to 4.48 ppm (peak b in Figure 1B) after the "click" copolymerization. These clearly confirm the formation of linkages between each PEG 2000 segment (see Supporting Information for ^{13}C NMR spectra of acetylene-terminated PEG 2000 and click PEG). SEC analysis of the product suggests that the resulting polymer (click PEG) has a high molecular weight (84.1 kDa) and a high polydispersity (PDI = 4.9). The polydispersity and the molecular weight of the polymer were hard to control when the ratio of propargyl amine (chain terminator) was adjusted.

To overcome this problem, acetylene-terminated monomethylether PEG 1900 (acetylene mPEG) was synthesized as a new chain terminator, which has a similar molecular weight to the acetylene-terminated PEG 2000. While the molecular weight of the click PEG can be controlled to some extent, the polydispersity of the polymer is still high (PDI $\approx$ 4).

The thermodynamic instability of Cu(I) is well-known because of its easy oxidation to Cu(II) and/or disproportionation to Cu(0) and Cu(II).[30] This could be another potential contributing factor to the high polydispersity of the click PEG. Inert atmospheres are recommended when Cu(I)-mediated processes are used. In addition, THPTA was synthesized as a stabilizing ligand for Cu(I) to protect it from oxidation and disproportionation.[27] We hypothesized that such a stabilizing ligand would enhance its catalytic activity and might, to a certain extent, control the polydispersity of the click PEG.

Evidently, the addition of THPTA to the "click" copolymerization does protect Cu(I) from oxidation and disproportionation. There is no Cu(0) precipitate even when the reaction was heated to 80 °C. Furthermore, the amount THPTA also affected the reaction rate of the "click" copolymerization. At 80 °C, the "click" copolymerization was rather slow when the THPTA/Cu(I) molar ratio was 100 with 1% catalyst. The majority of the overnight product was PEG 2000 dimer. When the THPTA/Cu(I) molar ratio was changed to 10 with 1% catalyst, the molecular weight of the product was very high and exceeded the detection limit of a Superdex 200 column. Lowering the temperature will slow down the reaction. At 21 °C, there was no reaction at all when the THPTA/Cu(I) molar ratio was 100 with 1% catalyst. With further optimization, we found the proper reaction conditions (1% catalyst, THPTA/Cu(I) = 10, 21 °C, as described in Experimental Section) that could produce linear multifunctional PEGs with much improved molecular weight and polydispersity (M_w = 23.7 kDa, PDI = 2.81, Figure 3). The terminal methoxy group of the product (from mPEG, peak g) is evident in its ^{1}H NMR spectrum (Figure 1B, see also the Supporting Information for its ^{13}C NMR spectrum).

To investigate the potential biomedical applications of this novel multifunctional PEG, we designed and synthesized a prototype bone-targeting drug delivery system. The strong bone affinity of bisphosphonates, including alendronate, has been well-documented.[31−33] To introduce osteotropicity into the delivery system, an alendronate-containing monomer (ALN-Azide) was synthesized to allow its incorporation into the linear multifunctional PEG via the "click" copolymerization. For the convenience of visualization and detection, a rhodamine-B-containing monomer (RB-Azide) was synthesized as a model drug component to be incorporated into the click PEG. These two monomers were reacted with acetylene PEG by a "click" reaction to obtain the new bone-targeting PEG conjugate (PEG-ALN-RB, M_w = 14.0 kDa, PDI = 2.40, Figure 3).

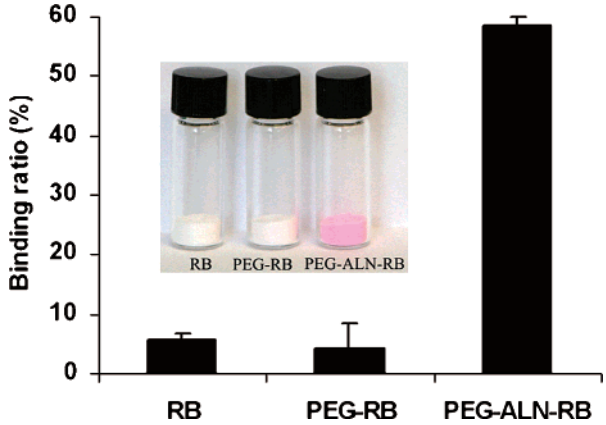


Figure 4. Binding of RB, rhodamine-B-labeled linear multifunctional PEG (PEG-RB), and rhodamine-B-labeled alendronate-containing linear multifunctional PEG (PEG-ALN-RB) to hydroxyapatite (HA) after 30 min of incubation. The inset shows the HA particles after incubation. They have been washed extensively with water and air-dried.

After dialysis and further purification with a Sephadex G-25 column, PEG-ALN-RB was analyzed with a UV/vis spectrophotometer. The peak at 218 nm belongs to the triazole structure of the click PEG. The peak at 565 nm is attributed to RB (model drug). The formation of a chromophoric complex between alendronate and Fe(III) ions in acidic media was applied to determine the amount of alendronate in the drug delivery systems using a UV/vis spectrophotometer (300 nm).[28] The model drug (RB) content was also determined using the ethyl ester of RB as the calibration standard. The results show that the alendronate content in PEG-ALN-RB is 40 μmol/g (91.9% conversion) and the RB content in the copolymer is 31.8 μmol/g (73.1% conversion) when 10% alendronate monomer, 10% RB monomer, and 80% 2,2-bis(azidomethyl)propane-1,3-diol were used in the "click" copolymerization.

Biomineral-Binding Ability of Click PEG. To investigate the bone-targeting potential of PEG-ALN-RB, its PBS solution was incubated with HA (model bone surface).[31] After incubation with HA, the PEG-ALN-RB that does not bind to HA (in the supernatant) was measured with a UV/vis spectrophotometer. In comparison to the original solution, the UV absorbance of the supernatant (at 565 nm) decreased to about 40% of the original after 30 min of incubation, which indicated that a large portion of the PEG-ALN-RB bound to the HA surface via the bisphosphonate moiety (Figure 4). However, the UV absorbance of PEG-RB and RB at 565 nm decreased only slightly, potentially due to their nonspecific binding to the HA surface. Repeated washing of the HAs with water yielded a white powder except for those treated with PEG-ALN-RB, which remained pink (Figure 4, inset). The binding of the conjugates to the surface of HA was very fast. The binding of PEG-ALN-RB almost reached a plateau in 5 min with about 50% of the conjugate bound to HA. Prolonged incubation of the PEG-ALN-RB with HA for 30 min led to an ultimate binding equilibrium of 60% bound. This outstanding HA-binding ability suggests a strong osteotropicity of the novel PEG conjugate in vivo and its potential applications in tissue-specific delivery of therapeutic agents to the skeleton.[31−33]

Conclusion

A linear, multifunctional PEG was synthesized by Huisgen 1,3-dipolar cycloaddition. Very simple building blocks and mild conditions were used for this copolymerization. A bone-targeting PEG conjugate based on this novel strategy was also successfully synthesized. The copolymerization is simple and yet highly efficient. In vitro studies indicated that the alendronate-containing PEG conjugate is a promising candidate for bone-targeting delivery of therapeutic agents. As the "click" reaction has no interference with other functional groups, additional pendant structures such as −COOH and $-NH_2$ may easily be introduced. Short segments of functional polymers (e.g., poly(*N*-isopropyl acrylamide), poly(lysine), or poly(acrylic acid)) may also be copolymerized with PEG segments to produce copolymers with unique biological and physicochemical properties. Evidently, this simple "click" copolymerization provides an effective tool for the development of novel functional polymers and polymeric conjugates for biomedical applications.

Acknowledgment. We thank Dr. Pavla Kopečková for providing PEG calibration standards for SEC analyses and the analytical method for the determination of alendronate content in the copolymers. We also acknowledge the financial support for this work from the College of Pharmacy of the University of Nebraska Medical Center.

Supporting Information Available. ^{13}C NMR spectra of acetylene-terminated PEG and click PEG. This information is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Chapman, A. P. *Adv. Drug Delivery Rev.* **2002**, *54*, 531−545.
(2) Greenwald, R. B.; Choe, Y. H.; McGuire, J.; Conover, C. D. *Adv. Drug Delivery Rev.* **2003**, *55*, 217−250.
(3) Duncan R. *Nat. Rev. Drug Discovery* **2003**, *2*, 347−360.
(4) Veronese, F. M.; Pasut, G. *Drug Discovery Today* **2005**, *10*, 1451−1458.
(5) Shen, L. J.; Shen, W. C. *Curr. Opin. Mol. Ther.* **2006**, *8*, 240−248.
(6) Kopeček, J.; Kopečková, P.; Minko, T.; Lu, Z. *Eur. J. Pharm. Biopharm.* **2000**, *50*, 61−81.
(7) Li, C. *Adv. Drug Delivery Rev.* **2002**, *54*, 695−713.
(8) Nathan, A.; Zalipsky, S.; Kohn, J. *J. Bioact. Compat. Polym.* **1994**, *9*, 239−251.
(9) Pechar, M.; Ulbrich, K.; Subr, V.; Seymour, L. W.; Schacht, E. H. *Bioconjugate Chem.* **2000**, *11*, 131−139.
(10) Cheng, J.; Khin, K. T.; Jensen, G. S.; Liu, A.; Davis, M. E. *Bioconjugate Chem.* **2003**, *14*, 1007−1017.
(11) Kumar, R.; Chen, M.-H.; Parmar, V. S.; Samuelson, L. A.; Kumar, J.; Nicolosi, R.; Yoganathan, S.; Watterson. A. C. *J. Am. Chem. Soc.* **2004**, *126*, 10640−10644.
(12) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004−2021.
(13) Lewis, W. G.; Green, L. G.; Grynszpan, F.; Radic, Z.; Carlier, P. R.; Taylor, P.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 1053−1057.
(14) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596−2599.
(15) Bock, V. D.; Hiemstra, H.; van Maarseveen, J. H. *Eur. J. Org. Chem.* **2006**, 51−68.
(16) Rodionov, V. O.; Fokin, V. V.; Finn, M. G. *Angew. Chem., Int. Ed.* **2005**, *44*, 2210−2215.
(17) Kolb, H. C.; Sharpless, K. B. *Drug Discovery Today* **2003**, *8*, 1128−1137.
(18) Manetsch, R.; Krasinski, A.; Radic, Z.; Raushel, J.; Taylor, P.; Sharpless, K. B.; Kolb, H. C. *J. Am. Chem. Soc.* **2004**, *126*, 12809−12818.
(19) Mocharla, V. P.; Colasson, B.; Lee, L. V.; Röper, S.; Sharpless, K. B.; Wong, C.-H.; Kolb, H. C. *Angew. Chem., Int. Ed.* **2005**, *44*, 116−120.
(20) Parrish, B.; Breitenkamp, R. B.; Emrick, T. *J. Am. Chem. Soc.* **2005**, *127*, 7404−7410.
(21) Malkoch, M.; Thibault, R. J.; Drockenmuller, E.; Messerschmidt, M.; Voit, B.; Russell, T. P.; Hawker, C. J. *J. Am. Chem. Soc.* **2005**, *127*, 14942−14949.
(22) Ladmiral, V.; Mantovani, G.; Clarkson, G. J.; Cauet, S.; Irwin, J. L.; Haddleton, D. M. *J. Am. Chem. Soc.* **2006**, *128*, 4823−4830.

(23) Wu, P.; Feldman, A. K.; Nugent, A. K.; Hawker, C. J.; Scheel, A.; Voit, B.; Pyun, J.; Fréchet, J. M. J.; Sharpless, K. B.; Fokin, V. V. *Angew. Chem., Int. Ed.* **2004**, *43*, 3928−3932.
(24) Joralemon, M. J.; O'Reilly, R. K.; Hawker, C. J.; Wooley, K. L. *J. Am. Chem. Soc.* **2005**, *127*, 16892−16899.
(25) Wang, Q.; Chan, T. R.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. *J. Am. Chem. Soc.* **2003**, *125*, 3192−3193.
(26) Beatty, K.; Xie, F.; Wang, Q.; Tirrell, D. A. *J. Am. Chem. Soc.* **2005**, *127*, 14150−14151.
(27) Chan, T. R.; Hilgraf, R.; Sharpless, K. B.; Fokin, V. V. *Org. Lett.* **2004**, *6*, 2853−2855.
(28) Kuljanin, J.; Jankovic, I.; Nedeljkovic, J.; Prstojevic, D.; Marinkovic, V. *J. Pharm. Biomed. Anal.* **2002**, *28*, 1215−1220.
(29) Odian, G. *Principles of Polymerization*, 4th ed.; Wiley-Interscience: Hoboken, NJ, 2004; pp 74−80.
(30) Simmons, M. G.; Merrill, C. L.; Wilson, L. J.; Bottomley, L. A.; Kadish, K. M. *J. Chem. Soc., Dalton Trans.* **1980**, 1827−1837.
(31) Wang, D.; Miller, S.; Sima, M.; Kopečková, P.; Kopeček, J. *Bioconjugate Chem.* **2003**, *14*, 853−859.
(32) Uludag, H.; Yang, J. *Biotechnol. Prog.* **2002**, *18*, 604−611.
(33) Zhang, S.; Gangal, G.; Uludag, H. *Chem. Soc. Rev.* **2007**, *36*, 507−531.

BM070430I