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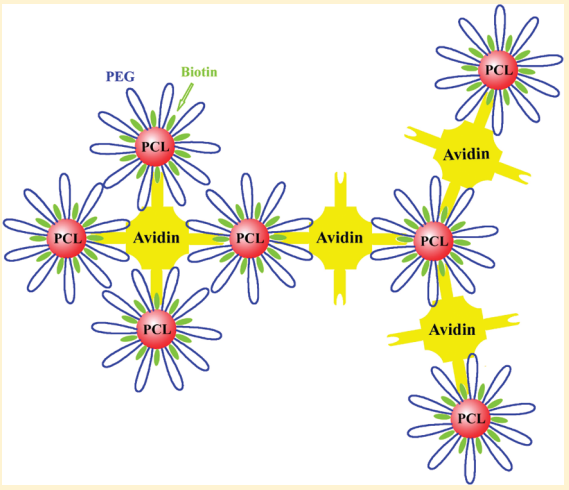
Amphiphilic Triblock Copolymer Bioconjugates with Biotin Groups at the Junction Points: Synthesis, Self-Assembly, and Bioactivity

Jie Jin,[†,§] Dongxia Wu,[†,§] Pingchuan Sun,[‡] Li Liu,[*,‡] and Hanying Zhao[*,†]

[†]Key Laboratory of Functional Polymer Materials, Ministry of Education, Department of Chemistry and [‡]Institute of Polymer Chemistry, Nankai University, Tianjin 300071, P. R. China

Supporting Information

ABSTRACT: Poly(ε-caprolactone)-*block*-poly(ethylene glycol)-*block*-poly(ε-caprolactone) (PCL-*b*-PEG-*b*-PCL) triblock copolymers with biotin groups at the junction points were synthesized based on a combination of click chemistry and ring-opening polymerization (ROP). Alkyne-functionalized PCL-*b*-PEG-*b*-PCL triblock copolymers were synthesized by using alkyne-functionalized PEG as macroinitiators in ROP of ε-caprolactone. Click chemistry was employed in the synthesis of the biotinylated triblock copolymers. Gel permeation chromatography and ^{1}H NMR results all indicated successful synthesis of well-defined triblock copolymers. The triblock copolymer chains can self-assemble into micelles in aqueous solution. The PCL blocks form the cores of the micelles and the hydrophilic PEG blocks form the coronae. The biotin moieties distribute at the interface of the micelles. Upon addition of avidin to the micellar solution, micelles aggregated together forming micellar aggregates due to the interaction between avidin and biotin. The avidin/HABA competitive binding assay also proved the bioavailability of the biotinylated micelles to avidin.



INTRODUCTION

Covalent conjugation to synthetic polymers is an efficient way to improve the stability, activity, and biocompatibility of many biomacromolecules. Bioconjugate polymers present virtues of both synthetic polymers and biomacromolecules and have found a variety of applications, including drug delivery,[1] biocatalysis,[2] and biosensing.[3]

Biodegradable and biocompatible aliphatic polyesters have received great attention over the past two decades. Among them, poly(ε-caprolactone) (PCL), poly(lactic acid), and their copolymers have been widely developed in medical reaserch.[4−7] PCL is a nontoxic biodegradable polymer, which can be biodegraded in the Krebs cycle into soluble nontoxic oligomers.[8] In the meanwhile, PCL is a hydrophobic and crystalline polymer, which considerably restrains its potential applications. Introduction of hydrophilic polyether blocks into PCL chains is a means to enhance the hydrophilicity as compared with the parent homopolymer. Poly(ethylene glycol) (PEG) is an uncharged, water-soluble, nontoxic, nonimmunogenic polymer and therefore an ideal material for bioapplications. In block copolymers of PEG and PCL (PEG-*b*-PCL), the PCL blocks present hydrophobic properties while PEG blocks present hydrophilic properties in the final materials. So block copolymers of PEG and PCL have been developed for their unique properties, such as biocompatibility, amphiphilicity, self-assembly, permeability, biodegradability, and crystallization properties.[9−12] But until now, the study on functionalization of PEG and PCL block copolymers is limited.

Biotin is a biomolecule that can bind to the enzyme avidin, yielding a stable complex with an association constant of 10^{15} mol^{-1}.[13] Avidin has four binding sites for biotin and can serve as a linkage between the biotinylated materials and other proteins. Recently, the biotin−avidin complex system was introduced to drug delivery systems for pretargeting purposes.[14] Biotinylated polymers have been known to undergo receptor-mediated endocutosis in both animal and plant cells.[15] Biodegradable biotinylated homopolymers[16] and block copolymers were synthesized by various approaches.[17] The Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reactions of azides and organic alkynes, also called "click" chemistry, were introduced by Sharpless et al.[18] Click reactions have gained a great deal of attention due to the excellent functional-group tolerance, high specificity, and nearly quantitative yields under mild experimental conditions.[19,20] Click reactions have been widely used as a versatile method in the synthesis of polymers with different architectures,[21−24] monomers,[25] and protein-based biohybrids.[26−28] In previous papers we reported preparation of biotinylated polymeric micelles by postpolymerization functionalization[29] and preparation of biotinylated Janus silica particles by in-situ click chemistry.[30]

In this paper, a combination of ring-opening polymerization (ROP) and 1,3-dipolar cycloaddition reactions of azides and

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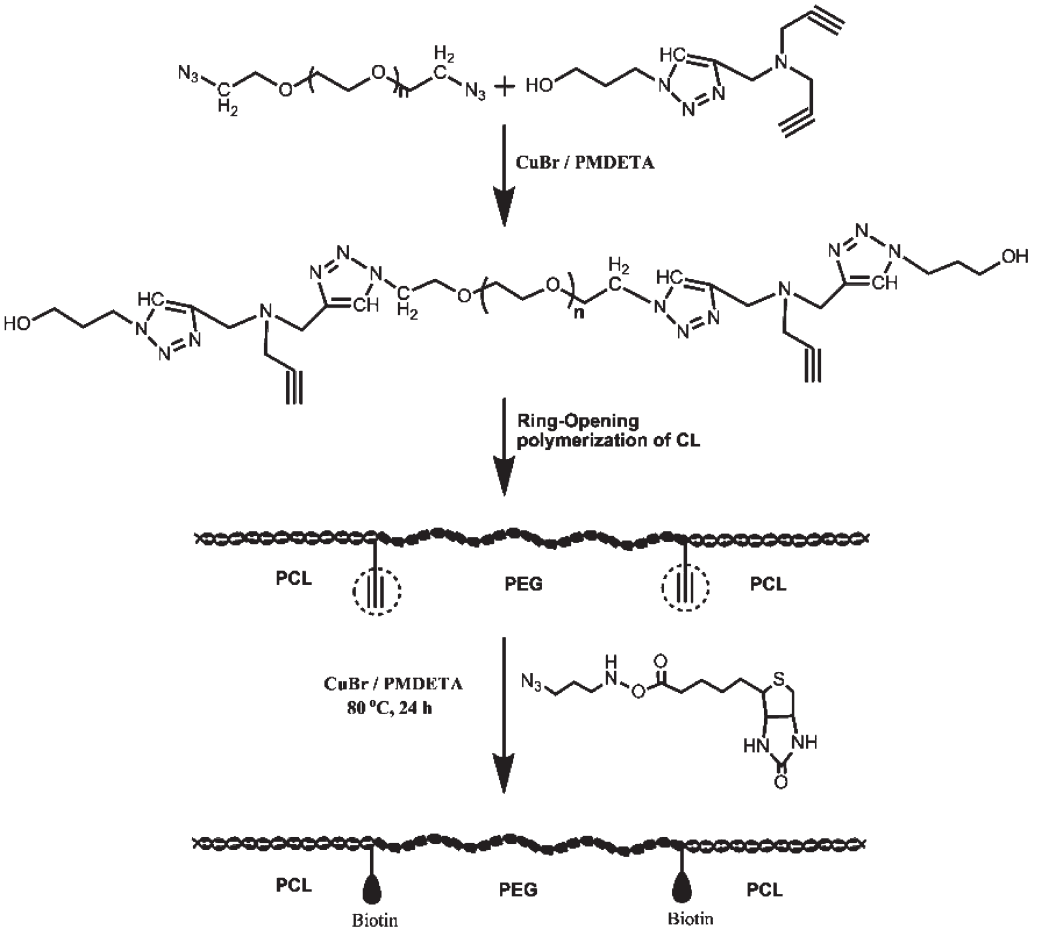
Scheme 1. Synthesis of Biotinylated Poly(ε-caprolactone)-*block*-poly(ethylene glycol)-*block*-poly(ε-caprolactone) (PCL-*b*-PEG-*b*-PCL) Triblock Copolymers Based on Click Chemistry and Ring-Opening Polymerization

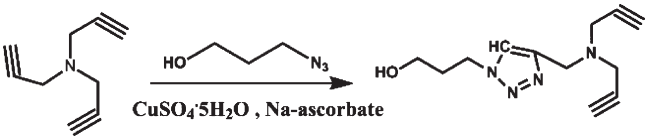


terminal alkynes was used as a strategy for preparation of PCL-*b*-PEG-*b*-PCL triblock copolymers with biotin groups at the junction points (Scheme 1). Alkynes functionalized PCL-*b*-PEG-*b*-PCL triblock copolymers were synthesized by using alkynes functionalized dihydroxyl PEG as macroinitiators for ROP of ε-caprolactone, and click chemistry was employed in the synthesis of biotinylated PCL-*b*-PEG-*b*-PCL triblock copolymers.

EXPERIMENTAL SECTION

Materials. *N*-Hydroxysuccinimide (NHS) (Fluka, 97%) was purified by recrystallization from a mixture of ethyl acetate and ethanol and dried under vacuum. ε-Caprolactone (Shanghai Hao Sheng Trade Co., 99%) was distilled before use. CuBr (99.5%) was purchased from Guo Yao Chemical Co. and was purified by washing with glacial acetic acid and dried in a vacuum oven at 100 °C. L-Ascorbic acid sodium salt (Alfa, 99%), 3-bromo-1-propanol (Alfa, 99%), sodium azide (Alfa, 99%), 3-chloropropylamine hydrocholoride (Alfa, 97%), biotin (Sigma), $CuSO_4 \cdot 5H_2O$ (Tianjin Rui Jin Chemical Co., 99%), EDC·HCl (Shanghai Medpep Co., Ltd., 99%), PEG with number-average molecular weight of 2000 g/mol (Tianjin Tian Tai Chemical Co., 98%), methanesulfonyl chloride (Tianjin Guang Fu Chemical Co., 99%), tripropargylamine (Aldrich, 98%), tin(II) 2-ethylhexanoate ($Sn(Oct)_2$, Aldrich, 95%), avidin/2-(4-hydroxyphenylazo)benzoic acid (HABA) reagent (Aldrich), and *N*,*N*,*N*′,*N*″,*N*″′-pentamethyldiethylenetriamine (PMDETA, Aldrich, 99%) were all used as received. All the solvents were distilled before use.

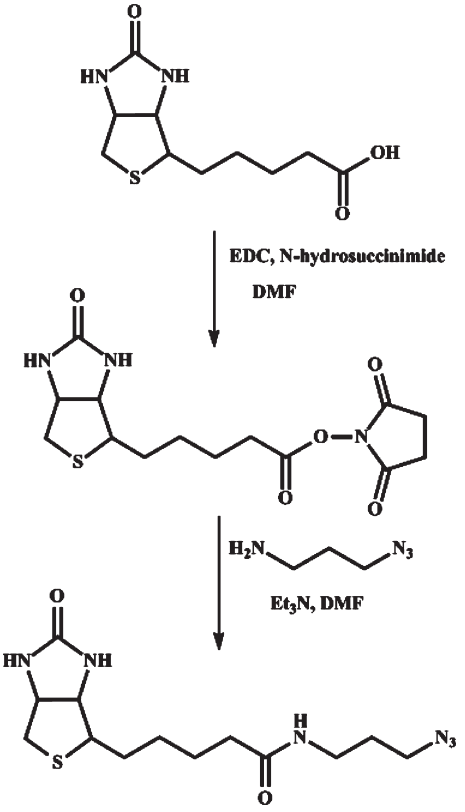
Scheme 2. Click Coupling Reaction of 3-Azidopropanol and Tripropargylamine



Preparation of 3-Azidopropanol. The synthesis of 3-azidopropanol was similar to previous literature.[31] Sodium azide (1.92 g, 30.0 mmol) and 3-bromo-1-propanol (2.5 g, 18 mmol) were added to a mixture of acetone (30 mL) and water (5 mL). The mixture was stirred at refluxed for 24 h. Acetone was removed on a rotary evaporator. The product was extracted with ether, dried over anhydrous magnesium sulfate ($MgSO_4$), and filtered, and the solvent was removed by evaporation. The product was dried under vacuum, and colorless oil was obtained (75% yield). ^{1}H NMR (400 MHz) in $CDCl_3$ (δ, ppm): 3.76 (t, 2H), 3.46 (t, 2H), and 1.84 (m, 2H).

Synthesis of 3-(4-((Dipropargylamino)methyl)-1,2,3-triazolyl)-propanol (DPAMTP). DPAMTP was synthesized by click reaction between 3-azidopropanol and tripropargylamine. The chemical reaction is shown in Scheme 2. The synthesis is described as follows. 3-Azidopropanol (0.10 g, 0.99 mmol) was dissolved in THF/water mixture (14 mL, 1:1, v/v), and tripropargylamine (0.220 mL, 1.55 mmol), $CuSO_4 \cdot 5H_2O$ (8.0 mg, 0.032 mmol), and sodium ascorbate (80.0 mg, 0.404 mmol) were added into

Scheme 3. Synthesis of Azido-biotin

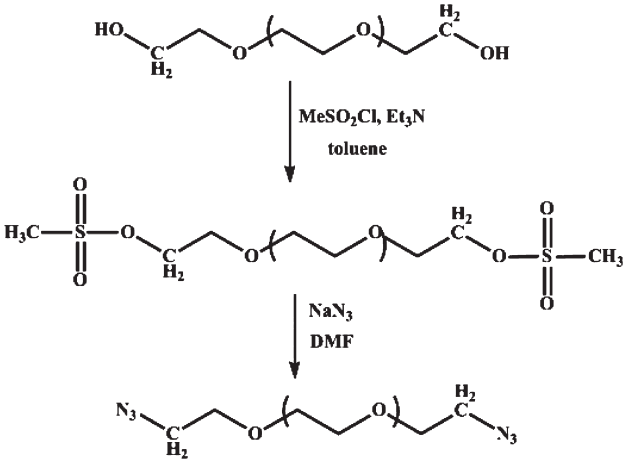


the solution. The yellow solution was stirred for 24 h at room temperature. The reaction mixture was evaporated under reduced pressure. The crude product was dissolved in CH_2Cl_2 and washed with water until pH of the solution reached 7.0. The organic layer was collected and dried with anhydrous $MgSO_4$. After filtration, the solvent was removed by rotary evaporation, and the product was dried under vacuum to yield yellow oil. The product was further purified by silica gel column chromatography with a mixture of petro ether/ethyl acetate (1:1, v/v) as the eluent (33% yield). ^{1}H NMR (400 MHz) in $CDCl_3$ (δ, ppm): 7.65 (s, 1H), 4.52 (t, 2H), 3.92 (s, 2H), 3.65 (t, 2H), 3.50 (s, 4H), 2.30 (s, 2H) and 2.13 (m, 2H). MS (chemical ionization): calcd for $C_{12}H_{17}N_4O$: 233.287. Found: 233.35.

Synthesis of Azido-biotin (Biotin-N_3). The synthesis of azido-biotin is shown in Scheme 3. 3-Azidopropylamine was synthesized according to previous literature.[32] To a solution of 3-chloropropylamine hydrocholoride (4.00 g, 0.0307 mol) in water (28 mL) at room temperature were added NaN_3 (10.0 g, 0.154 mol) and a pinch of KI. The reaction mixture was stirred at 90 °C for 72 h. The reaction solution was cooled to room temperature, and NaOH was added until pH reached 11. Solid NaCl was then added, and the mixture was extracted five times with toluene. The combined organic layers were dried with anhydrous $MgSO_4$. After filtration, toluene was partially removed under reduced pressure on a rotary evaporator at a low temperature. On the basis of ^{1}H NMR result, the concentration of the 3-azidopropylamine in the resulting toluene solution was about 2.4 mmol/mL.

In order to synthesize biotin-NHS, EDC (245 mg, 1.28 mmol) was added to a mixture of biotin (0.293 g, 1.20 mmol) and NHS (151 mg, 1.32 mmol) in 22 mL of dry *N*,*N*-dimethylformamide (DMF).[33] After 24 h stirring at room temperature, the reaction solution was concentrated to yield a white solid. The product was washed by methanol and dried under vacuum, and a white solid was obtained with 69% yield. ^{1}H NMR (400 MHz) in DMSO-d_6 (δ, ppm): 6.42, 6.36 (d, 2H), 4.30, 4.15 (m, 2H), 3.11 (m, 1H), 2.86 (d, 1H), 2.82 (s, 4H), 2.67 (t, 2H), 2.60 (d, 1H), 1.71−1.34 (m, 6H).

Scheme 4. Synthesis of Azido-Terminated PEG (N_3-PEG-N_3)



The synthesis of biotin-N_3 is described as follows. To a solution of biotin-NHS (0.14 g, 0.41 mmol) and triethylamine (0.12 mL, 0.86 mmol) in dry DMF (16 mL) was slowly added 3-azidopropylamine (80 mg, 0.80 mmol). After 24 h stirring at room temperature, the reaction solution was concentrated under reduced pressure, and a solid product was obtained. After washing with water, the crude product was purified by gel column chromatography ($MeOH:CHCl_3$:AcOH = 8:1:0.05) to give the desired biotin-N_3 (100 mg, 77% yield). ^{1}H NMR (400 MHz) in DMSO-d_6 (δ, ppm): 7.84 (t, 1H), 6.42, 6.36 (d, 2H), 4.30, 4.13 (m, 2H), 3.33 (t, 2H), 3.10 (m, 3H), 2.86 (d, 1H), 2.60 (d, 1H), 2.06 (q, 2H), 1.69−1.20 (m, 8H).

Synthesis of Azido-Terminated PEG (N_3-PEG-N_3). Azido-terminated PEG was synthesized by using a procedure similar to previous literature.[21] A typical procedure for the synthesis of azido-terminated PEG is briefly described as follows (Scheme 4). In a dry round-bottom flask, HO-PEG-OH (10 g, 5.0 mmol) with number-average molecular weight of 2000 was dissolved in 60 mL of toluene, and triethylamine (7 mL, 0.05 mol) was added into the solution. The solution was then cooled to 0 °C, methanesulfonyl chloride (3.0 mL, 0.038 mol) was added dropwise, and the solution was stirred at room temperature for 24 h. The solvent was removed under reduced pressure. The solid was then dissolved in CH_2Cl_2 and washed successfully with 1 M HCl solution, 1 M NaOH solution, and 1 M NaCl solution. The organic layer was dried over anhydrous $MgSO_4$ overnight, precipitated in cold ether. After filtration, the yield mesylate-terminated polymer (CH_3O_2SO-PEG-OSO_2CH_3) was dried under vacuum. Part of the mesylate-terminated PEG (3.0 g, 0.0015 mol) was dissolved in 30 mL of dry DMF; sodium azide (0.48 g, 0.0075 mol) was then added. The reaction mixture was stirred magnetically at 50 °C for 24 h. After reaction DMF was removed by rotary evaporation. The solid was dissolved in methylene chloride, and the undissolved solid was removed by filtration. Before dried over anhydrous $MgSO_4$ overnight, the organic solution was washed twice by water. After concentration of the organic solution on a rotatory evaporator, azido-terminated PEG was precipitated in cold ether. After filtration and drying under vacuum, dry polymer was obtained. ^{1}H NMR (400 MHz) in $CDCl_3$ (δ, ppm), CH_3O_2SO-PEG-OSO_2CH_3: 3.08 (s, 6H), 3.43−3.84 (m, 172H), 4.37 (t, 4H). N_3−PEG-N_3: 3.42−3.84 (m, 172H), 3.37 (t, 4H).

Click Reaction between Azido-Terminated PEG and DPAMTP. PEG macroinitiator with one hydroxyl group and one alkyne group at each end, assigned as HA-PEG in this research, was synthesized by "click" reaction between azido-terminated PEG and DPAMTP. A typical reaction is described as follows. N_3-PEG-N_3 (0.50 g, 0.25 mmol) and DPAMTP (1.16 g, 5.0 mmol) were dissolved in 4 mL of dry DMF in a 25 mL Schlenk flask, and PMDETA (53 μL, 0.25 mmol) was added. After the mixture was bubbled with nitrogen for 15 min, CuBr (36 mg, 0.25 mmol)

was added. The solution was degassed by three freeze−pump−thaw cycles. The coupling reaction was carried out at 35 °C for 48 h and stopped by exposure of the solution to air. Copper ions were removed from the polymer solution with neutral alumina column chromatography. The product was isolated by precipitation into cold diethyl ether. After filtration, the polymer was dried under vacuum.

Synthesis of PCL-*b*-PEG-*b*-PCL Triblock Copolymers by Ring-Opening Polymerization. HA-PEG was used as a macroinitiator in ROP of CL. The chain length of PCL in the triblock copolymers was controlled by controlling the monomer/macroinitiator ratio or monomer conversion. A typical polymerization is described as follows. HA-PEG (30 mg, 0.015 mmol) and ε-caprolactone (0.30 mL, 2.8 mmol) were dissolved in 1 mL of dry DMF in a 10 mL Schlenk flask, and the mixture was bubbled with nitrogen for 15 min. After 15 min stirring, $Sn(Oct)_2$ (5.0 mg, 0.012 mmol) was added, and the solution was degassed by three freeze−pump−thaw cycles. The polymerization was conducted at 110 °C for 24 h and stopped by exposure to air. The polymer solution was precipitated in cold methanol. After filtration, the polymer was dried under vacuum.

Synthesis of PCL-*b*-PEG-*b*-PCL Triblock Copolymers with Biotins at the Junction Points. Biotin-N_3 (11.7 mg, 0.0360 mmol) and PCL-*b*-PEG-*b*-PCL (67.1 mg) were mixed in 0.5 mL of dry DMF in a 10 mL Schlenk flask; PMDETA (63 μL, 0.30 mmol) and CuBr (44 mg, 0.30 mmol) were added after the mixture was bubbled with nitrogen for 15 min. The solution was degassed by three freeze−pump−thaw cycles. The reaction was conducted at 80 °C for 24 h and stopped by exposure to air. The polymer solution was dialyzed against aqueous phosphate buffer solution (PBS, pH = 8.0, 0.05 M) and doubly distilled water to remove excess biotin-N_3 and other small molecules. The biotinylated triblock copolymers were obtained after freeze-drying.

Avidin-HABA Assay Studies. The amount of available biotin on micelles was determined by avidin/HABA reagent. The avidin/HABA reagent was reconstituted with 10 mL of deionized water. In a 1 mL cuvette, 900 μL of HABA/avidin reagent was pipetted, and the absorbance was measured at λ = 500 nm ($A_{500}^{\mathrm{HABA/Avidin}}$) by a UV−vis spectrophotometer. To this solution, 100 μL of sample was added, the solution was mixed by inversion, and the absorbance at λ = 500 nm ($A_{500}^{\mathrm{HABA/Avidin+Sample}}$) was read. The amount of the available biotin was calculated by the following formula:[34,35] μmol biotin/mL = ($\Delta A500/34$) × 10, which corresponds to the micromoles of biotin per milliliter of the sample solution, where $\Delta A500 = 0.9(A_{500}^{\mathrm{HABA/Avidin}}) - A_{500}^{\mathrm{HABA/Avidin+Sample}}$.

Preparation of Polymer Micelles. Biotinylated PCL-*b*-PEG-*b*-PCL triblock copolymer was dissolved in THF, and the solution was added dropwise to 7-fold of doubly distilled water forming a cloudy solution. After stirring for 1 day, the cloudy micellar solution was transferred to a dialysis tubing (MWCO 7 kDa) and dialyzed against doubly distilled water for 2 days.

Characterization. ^{1}H NMR spectra were recorded on a Varian 400 spectrometer. Biotin-NHS and biotin-N_3 were measured in deuterated DMSO, and other products were measured in deuterated chloroform. The apparent molecular weight and molecular weight distributions of the PCL-*b*-PEG-*b*-PCL triblock copolymers were determined with a gel permeation chromatograph (GPC) equipped with a Waters 717 autosampler, a Waters 1525 HPLC pump, three waters UltraStyragel columns with 5K−600 K, 500K−30K, and 100K−10K molecular ranges, and a Waters 2414 refractive index detector. THF was used as eluent at a flow rate of 1.0 mL/min. Molecular weights were calibrated on PS standards. Fourier transform infrared absorption spectra (FTIR) were obtained on a Bio-Rad FTS6000 system using diffuse reflectance sampling accessories. Transmission electron microscopy (TEM) observations were carried out on a Tecnai G2 20 S-TWIN electron microscope equipped with a Model 794 CCD camera. TEM specimens were prepared by dipping copper grids into the micellar solution (about 0.25 mg/mL) and drying in air. UV−vis absorption spectra were collected on a JASCO V-570 spectrometer. Dynamic light scattering (DLS) measurements were conducted on a Zetasizer Nano ZS from Malvern Instruments equipped with a 10 mW HeNe laser at a wavelength of 633 nm. The results were analyzed in CONTIN mode. Mass spectrometry was performed on a Finnigan LCQ advantage ion-trap mass spectrometer. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a Bruker Autoflex III spectrometer equipped with a 337 nm nitrogen laser. α-Cyano-4-hydroxycinnamic acid was used as the matrix. Mass spectra were acquired in reflex mode at an acceleration voltage of +20 kV.

RESULTS AND DISCUSSION

As shown in Scheme 1, three steps are involved in the synthesis of biotinylated triblock copolymers. At the first step, HA-PEG with one hydroxyl and one alkyne group at each end was synthesized by coupling reaction of azide-terminated PEG and DPAMTP. At the second step, hydroxyl groups at the ends of HA-PEG chains were used to initiate ROP of ε-caprolactone, and amphiphilic PCL-*b*-PEG-*b*-PCL triblock copolymers with alkyne groups at the junction points were prepared. On one block copolymer chain there are two alkyne groups at the junction points. At the last step, biotin groups were grafted to the junction points of PCL-*b*-PEG-*b*-PCL triblock copolymer chains via click reaction.

3-Azidopropanol was prepared by reaction of 3-bromo-1-propanol and NaN_3 in dry DMF, and DPAMTP was synthesized by click reaction between 3-azidopropanol and tripropargylamine (Scheme 2). ^{1}H NMR spectra of 3-azidopropanol and DPAMTP are shown in Figure S1 (Supporting Information). The signals of 3-azidopropanol at 3.76, 3.46, and 1.84 ppm represent the methylene protons next to the hydroxyl group (peak a), the methylene protons next to the azide group (peak b), and the middle methylene protons (peak c), respectively (spectrum a in Figure S1). After click reaction with tripropargylamine, DPAMTP was obtained. The signals of DPAMTP (spectrum b in Figure S1) at 7.65 ppm representing the proton on the 1,2,3-triazole ring (g), at 4.52 ppm representing the methylene protons next to the ring (b′), at 3.92 ppm representing the methylene protons between the nitrogen atom and the 1,2,3-triazole ring (e), at 3.50 ppm representing the methylene protons next to the alkyne groups (f), and at 2.30 ppm representing the protons on alkyne groups (peak d) are observed. The integral ratio of g:b′:e:f:d is 1:2:2:4:2, which indicates the successful synthesis of DPAMTP. The molecular mass of DPAMTP measured by MS (MH^+, chemical ionization) is 233.35, which is very close to the theoretic value at 233.28.

N_3-PEG-N_3 was synthesized by a reaction of HO-PEG-OH with methanesulfonyl chloride and followed by transformation of mesylate end groups to azido groups via a reaction with NaN_3 in dry DMF (Scheme 4). ^{1}H NMR was used to characterize chemical structures of the obtained polymers. ^{1}H NMR spectra of mesylate-terminated (spectrum a) and azido-terminated PEG (spectrum b) are shown in Figure S2 (Supporting Information). In the spectrum of mesylate-terminated PEG, the peak at 3.08 ppm represents the methyl protons at mesylate end groups, and the peak at 4.37 ppm represents the methylene protons next to the ester groups. The integral ratio of peak a to peak b is 3:2, which indicates that the esterification reaction is quantitative. After a reaction with NaN_3, the signals of mesylate-terminated PEG at 3.08 and 4.37 ppm disappeared completely, and a new peak at 3.37 ppm representing the methylene protons next to the azido groups is observed in the ^{1}H NMR spectrum of the azide chain-end-modified PEG.

Linear HA-PEG with one hydroxyl group and one alkyne group at each end was synthesized by click coupling reaction between N_3-PEG-N_3 and DPAMTP. In multistep click reactions side products may be produced.[36] Two N_3-PEG-N_3 macromolecules may react

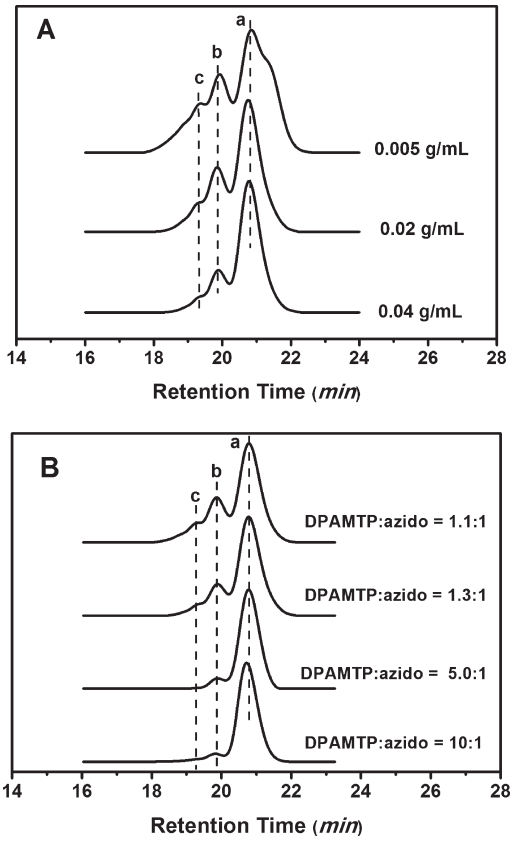


Figure 1. (A) GPC elution chromatograms of PEG with one hydroxyl group and one alkyne group at each end (HA-PEG) prepared at different polymer concentrations. The molar ratio of 3-(4-((dipropargylamino)-methyl)-1,2,3-triazolyl)propanol (DPAMTP) to azido groups is 1.3:1 in these reactions. (B) GPC elution chromatograms of HA-PEG prepared at different molar ratios of DPAMTP to azido groups. The concentration of N_3-PEG-N_3 is 4×10^{-2} g/mL in these reactions.

with one DPAMTP, producing the coupling product. The PEG coupled polymer may further react with DPAMTP and N_3-PEG-N_3, and macromolecules with three PEG segments in a polymer chain structure may be produced. Besides target product HA-PEG, macromolecules with two or three PEG segments in a structure are possibly produced. In order to investigate possible side reactions in the click reactions and optimize reaction conditions, controlled experiments were conducted. In the experiments, the concentration of N_3-PEG-N_3 or the molar ratio of azido to alkyne was controlled while the other reaction conditions were kept unchanged. Figure 1a shows the GPC chromatograms of the products prepared at different polymer concentrations. On each GPC curve, three peaks can be observed. On the basis of Figure 1, it is reasonable to expect that peak a with a peak molecular weight of 3.5K represents our target product HA-PEG, peak b with a peak molecular weight of 6.5K represents a polymer with two PEG segments in a chain, and peak c with a peak molecular weight of 9.9K represents a polymer with three PEG segments in a chian. All the three GPC curves in Figure 1a were normalized at retention time of 20.8 min (the position of peak a). The intensities of peaks b and c decreased with the increase of polymer concentration, indicating that there were less side reactions at higher polymer concentrations. The production of all the side products is related to the coupling reactions between two polymer chains. At a high polymer concentration click coupling reaction between two polymer chains is unfavorable due to

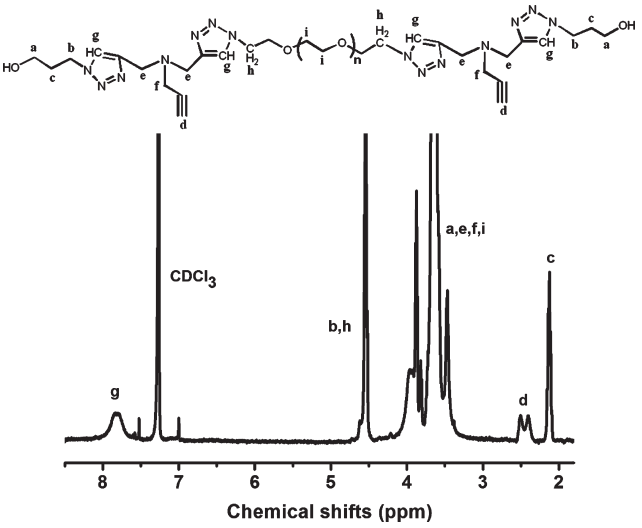


Figure 2. ^{1}H NMR spectrum of HA-PEG.

the high viscosity in the solution, and coupling reaction between a polymer chain and a small molecule is favorable. So the yield of HA-PEG is higher at a higher polymer concentration. Integration results of these peaks provided quantitative information about the fraction of different species. When the concentration of N_3-PEG-N_3 was 5×10^{-3}, 2×10^{-2}, and 4×10^{-2} g/mL, the fraction of HA-PEG was 0.65, 0.69, and 0.80, respectively.

The influence of the molar ratio of alkyne to azido groups on the synthesis of HA-PEG was also investigated in this research. Keeping the concentration of N_3-PEG-N_3 at 4×10^{-2} g/mL, HA-PEG was prepared at different molar ratios of DPAMTP to azido groups. Figure 1b shows the GPC chromatograms of polymers prepared at different molar ratios. When the molar ratio was 1.1:1 or 1.3:1, three peaks were observed on the GPC curves. However, when the molar ratio reached 5.0:1, peak c disappeared. With further increase of the molar ratio, the intensity of peak b became very smaller. The fractions of HA-PEG in the products prepared at molar ratios of 5.0:1 and 10:1 were 0.94 and 0.96, respectively. High purity target polymer was prepared at high molar ratio of DPAMTP to azido groups. Excess DPAMTP was removed by repeated dissolving and precipitation cycles using system of dichloromethane/diethyl ether (solvent and precipitant for PEG). GPC curves proved that after four dissolving and precipitation cycles excess DPAMTP was removed completely (Figure S3 in Supporting Information).

Figure 2 shows the ^{1}H NMR spectrum of HA-PEG. In Figure 2 peaks at 3.23−4.20 ppm represent the overlapped signals of oxymethylene protons (−OCH_2CH_2−) in PEG (i, δ = 3.65 ppm), methylene protons −NCH_2C− (e, δ = 3.92 ppm), −CCH_2O− (a, δ = 3.65 ppm), and −CCH_2N− (f, δ = 3.50 ppm) in DPAMTP structure. In the spectrum signals at 7.78 ppm (peak g) representing the −CH− proton on the triazole ring, at 2.15 ppm (peak c) representing the methylene protons at terminal ends, and at 2.30 ppm (peak d) representing the protons on alkyne groups were also observed. The integral ratio of peak g to c to d is about 2:2:1, which indicates there is one alkyne group and one hydroxyl group at each end, and hence proves successful "click" reaction between DPAMTP and N_3-PEG-N_3 (Scheme 1). MALDI-TOF MS of HA-PEG was also measured to prove the target polymer structure. As shown in Figure 3, there are two series of repeating peaks, both of which have an interval of 44, representing the molecular weight of ethylene oxide unit. The molecular formula of HA-PEG is $44n + 274 \times 2 + 28$,

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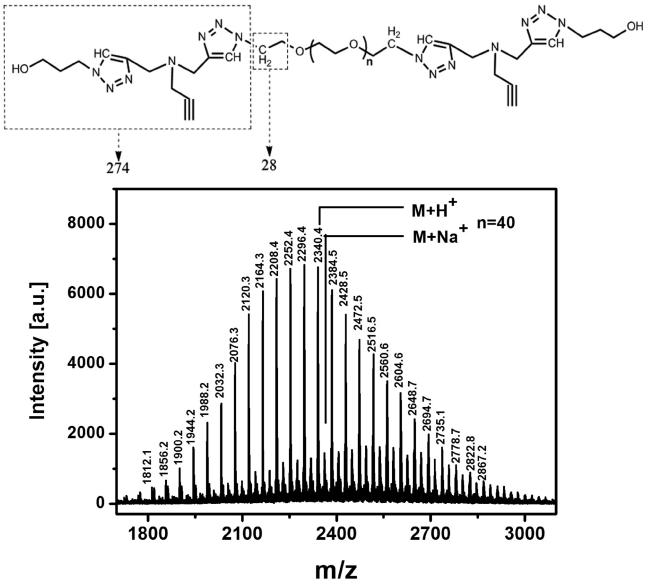

Figure 3. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrum of HA-PEG. $M = 44n + 274 \times 2 + 28$. Matrix: α-cyano-4-hydroxycinnamic acid; cation source: H^+ and Na^+ in glassware, solvents, and reagents.

where n is 40. The two series of repeating peaks are attributed to HA-PEG plus H^+ and Na^+. The molecular mass for HA-PEG plus H^+ and HA-PEG plus Na^+ were calculated to be 2338.53 and 2360.53, respectively, which agreed well with the results of MALDI-TOF MS (2340.4 and 2362.4). This result confirmed the success of click reaction between N_3-PEG-N_3 and DPAMTP and high purity of the target product HA-PEG.

The hydroxyl groups at two ends of a HA-PEG chain were used to initiate ROP of ε-caprolactone, and PCL-*b*-PEG-*b*-PCL triblock copolymers with alkyne groups at the juntion points were prepared. In this research a series of PCL-*b*-PEG-*b*-PCL triblock copolymers were synthesized by using $Sn(Oct)_2$ as catalyst, and the triblock copolymers were assigned as PCL_n-*b*-PEG-*b*-PCL_n, where n is the average polymerization degree of PCL blocks. GPC elution chromatograms of N_3-PEG-N_3, HA-PEG, and PCL_{75}-*b*-PEG-*b*-PCL_{75} are shown in Figure 4. After ROP, the GPC curve of the triblock copolymer moves to high molecular weight part. It is also noted that the molecular weight distribution of the triblock copolymer (PDI = 1.40) is broader than the two precursors, HA-PEG and N_3-PEG-N_3. The ^{1}H NMR spectrum of PCL_{20}-*b*-PEG-*b*-PCL_{20} triblock copolymer is shown in Figure 5a. In the spectrum, the peaks at 4.06 ppm (f) and 3.65 ppm (a) represent the methylene protons in PCL block and oxymethylene protons $-OCHH_2CH_2-$ in PEG block, respectively. By using the integral ratio of the two peaks, the molar ratio of PCL to PEG can be obtained. The number-average degree of polymerization obtained by ^{1}H NMR, number-average molecular weights, and molecular weight distributions of all the triblock copolymers measured by GPC are summarized in Table 1.

It is well-known that biotin binds with high affinity to avidin and streptavidin. The avidin−biotin interaction is the strongest known noncovalent biological recognition.[37,38] To investigate the application of the triblock copolymers in bioconjugation, PCL-*b*-PEG-*b*-PCL triblock copolymers with alkyne groups at the junction points were functionalized with biotin-N_3 by "click" reaction to obtain biotinylated PCL-*b*-PEG-*b*-PCL triblock copolymers. Scheme 3 illustrates the synthesis of biotin-N_3. After click reaction excess

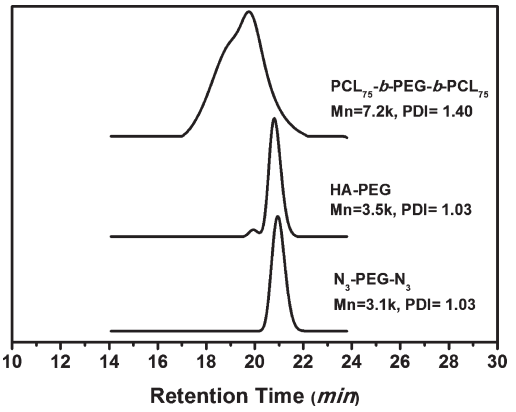

Figure 4. GPC elution chromatograms of the azide chain-end-modified PEG (N_3-PEG-N_3), HA-PEG, and PCL_{75}-*b*-PEG-*b*-PCL_{75} triblock copolymer.

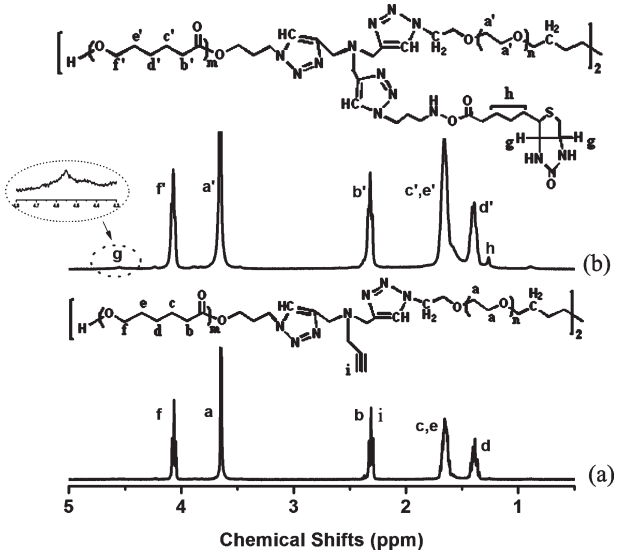

Figure 5. ^{1}H NMR spectra of triblock copolymer with alkyne groups at the junction points (a) and biotinylated triblock copolymer (b).

biotin-N_3 was removed by dialysis in aqueous PBS solution, and the biotin-functionalized triblock copolymers were lyophilized.

^{1}H NMR spectrum of biotin-functionalized PCL_{20}-*b*-PEG-*b*-PCL_{20} triblock copolymers is shown in Figure 5b. After click reaction, featured signals of biotin molecules were observed. In the ^{1}H NMR spectrum of biotinylated PCL_{20}-*b*-PEG-*b*-PCL_{20} triblock copolymer, the signal at 4.56 ppm (g) represents $-NHCHCH_2S-$ protons in biotin molecules, and the signal at 1.26 ppm (h) represents the methylene protons next to the biotin rings (Figure 5b). Because the signal at 4.06 ppm (f′) represents the methylene protons in PCL block and the signal at 3.65 ppm (a′) represents oxymethylene protons in PEG block, the average number of biotin molecules in a triblock copolymer chain can be calculated based on the integral ratio of peak g to f′ to a′. Our calculation result shows that the ratio is about 4:82:174, which is very close to the theoretical value. This result proved the successful click reaction between biotin-N_3 and PCL-*b*-PEG-*b*-PCL triblock copolymers with alkyne groups at the junction points.

Previous researches indicate that triblock copolymers can form micelles in solvents that preferentially dissolve the middle block.[39] For example, spherical micelles formed by BAB triblock

Table 1. Summary of Molecular Weights and Molecular Weight Distributions of PCL-*b*-PEG-*b*-PCL Triblock Copolymers

samples	average repeating unit number of PCL[a]	number-average molecular weights ($M_n \times 10^{-3}$)[b]	polydispersity[b]
PCL_{20}-*b*-PEG-*b*-PCL_{20}	20	4.6	1.67
PCL_{50}-*b*-PEG-*b*-PCL_{50}	50	6.0	1.50
PCL_{75}-*b*-PEG-*b*-PCL_{75}	75	7.2	1.40
PCL_{130}-*b*-PEG-*b*-PCL_{130}	130	10.0	1.62

[a] Measured by ^{1}H NMR. [b] Measured by GPC.

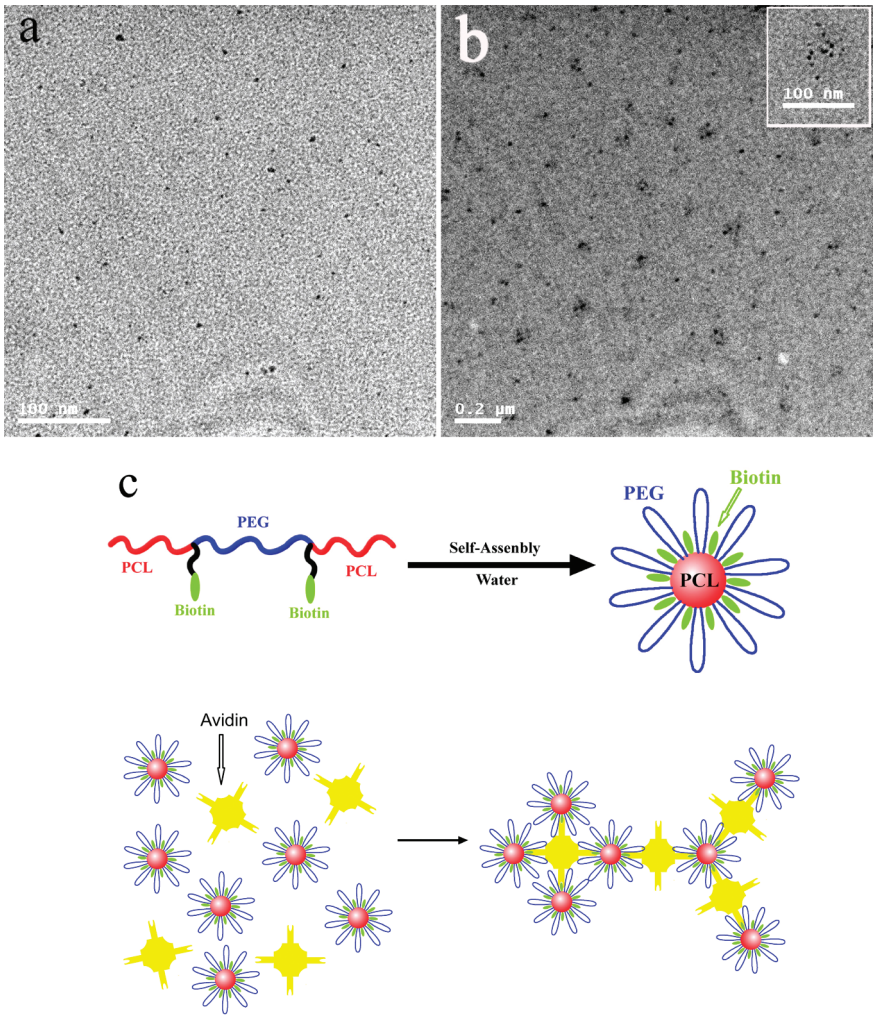

Figure 6. TEM images of self-assembly aggregates of biotinylated PCL_{20}-*b*-PEG-*b*-PCL_{20} triblock copolymer in aqueous solution before (a) and after addition of avidin (b) and schematic representations for the interaction of biotinylated triblock copolymer micelles with avidin (c).

copolymers in solvents that preferentially dissolve A block, would consist of a core containing B blocks, surrounded by loops of A blocks. The self-assembly of amphiphilic biotinylated PCL_{20}-*b*-PEG-*b*-PCL_{20} triblock copolymer in aqueous solution was investigated in this research. The triblock copolymer was dissolved in THF, and the THF solution was added dropwise to 7-fold of double-distilled water under vigorous stirring forming a cloudy solution. The solution was dialyzed against double-distilled water for 2 days to remove THF. TEM observations were conducted to study the structure of the self-assembled aggregates. The TEM specimens were stained in hydrazine atmosphere for 8 h and then in OsO_4 atmosphere for 100 min, and thus PCL blocks together with biotin molecules at the junction points were stained.[29] Figure 6a shows a TEM image of self-assembly of the triblock copolymer in aqueous solution. The triblock copolymer chains self-assembled into micellar structures in aqueous solution. The average size of the spherical dots representing micellar cores is about 10 nm. According to DLS results, the average hydrodynamic diameter of the micelles is about 35 nm (Figure 7). In aqueous solution, the collapsed hydrophobic PCL blocks constitute the cores of the micelles to avoid unfavorable interaction with water, while the hydrophilic PEG blocks form the coronae to stabilize the structures. In a triblock copolymer chain, the PEG block connects two PCL blocks at two ends. In order to bring two hydrophobic blocks into the core of a micelle, the hydrophilic PEG block must form a loop structure in the corona of a micelle. The functional biotin groups at the junction points of the triblock

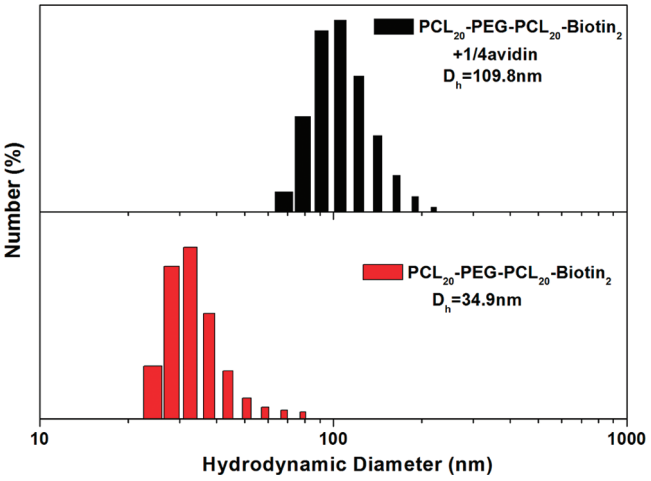

Figure 7. Size distributions of self-assembly of biotinylated PCL_{20}-*b*-PEG-*b*-PCL_{20} triblock copolymer in aqueous solutions before (below) and after addition of avidin (upper).

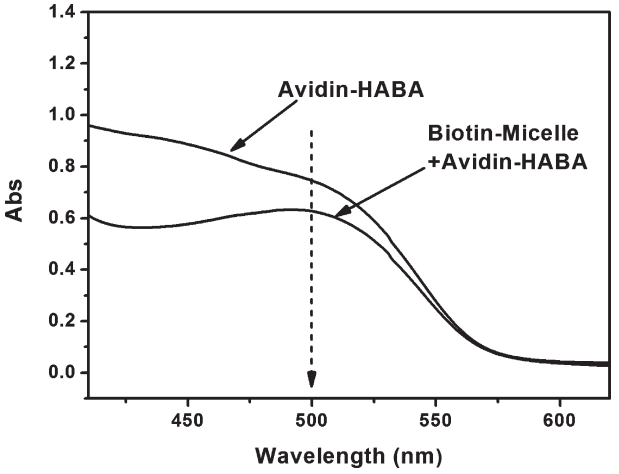

Figure 8. UV spectra of avidin−HABA complex before and after addition of biotinylated PCL-*b*-PEG-*b*-PCL triblock copolymer micelles.

copolymers reside at the interface of the micelles. The structure is shown in Figure 6c.

The interaction of biotin with avidin was proved by TEM. A solution of biotinylated triblock copolymer micelles was incubated with avidin (biotin/avidin = 4:1 molar ratio) for 10 h. On the TEM image aggregation of micelles were observed (Figure 6b), which indicated that biotin molecules on the different micelles binded to the same avidin molecule, and the addition of avidin to the biotinylated micelles induces the aggregation of micelles. A magnified TEM image was shown in the inset of Figure 6b. The structure of aggregated micelles was also shown in Figure 6c.

The hydrodynamic diameters of biotinylated triblock copolymer micelles before and after addition of avidin were measured by DLS. From the size distribution curve shown in Figure 7, it can be seen that before addition of avidin the block copolymer micelles possess a unimodal size distribution with a hydrodynamic diameter range from 25 to 80 nm. After addition of avidin, the size distribution moves to big size part with a size range from 70 to 220 nm. The change in size is due to the binding of avidin to the biotin molecules at the interfaces of micelles and the production of micellar aggregates.

The bioavailability of biotin at the interfaces of micelles to avidin was evaluated by an avidin/HABA competitive binding assay. It is known that the binding of HABA to avidin gives an absorption maximum at 500 nm. However, when biotin or biotinylated micelles are added, biotin molecules displace HABA from the complex with avidin, and the absorbance at 500 nm decreases. The absorption decrease is proportional to the concentration of biotin in the solution, and the change in absorbance can be used to calculate the amount of biotin. The assay was conducted by measuring the absorbance of the avidin/HABA complex at 500 nm before and after mixing with the biotinylated micellar solutions. Figure 8 shows UV−vis spectra of the avidin−HABA complex before and after addition of biotinylated PCL_{20}-*b*-PEG-*b*-PCL_{20} triblock copolymer micellar solution. Upon addition of the biotinylated micelles, the absorbance of the avidin/HABA complex at 500 nm decreased from 0.746 to 0.627, indicating that the biotin molecules in the polymeric micelles competitively bind to avidin by replacing HABA molecules. The amount of available biotin to avidin was calculated to be 12.7 nmol/mg polymer. It is worthy of note that this value is much lower than ^{1}H NMR result (260 nmol/mg polymer). The lower accessibility of biotin molecules to avidin can be explained by the fact that some biotin molecules at the interfaces of micelles could not interact with avidin because of the interface rigidity.[29,30,34] Additionally, avidin has four biotin binding sites, and it can bind biotin molecules on different polymeric micelles inducing the formation of micelle aggregates, which would also sterically prohibit interaction of biotin molecules with avidin.

CONCLUSIONS

In summary, PCL-*b*-PEG-*b*-PCL triblock copolymers with biotin groups at the junction points were synthesized by click chemistry and ring-opening polymerization. The triblock copolymers can self-assemble into micelles in aqueous solution. The hydrophobic PCL blocks form the cores of micelles, the hydrophilic PEG blocks form the coronae, and the biotin moieties distribute at the interfaces of micelles. The avidin/HABA competitive binding assay proved the bioavailability of the biotinylated micelles to avidin. Upon addition of avidin molecules to the micellar solutions, the aggregation of micelles was observed due to the interaction between biotin and avidin. This type of polymeric micelles could find potential applications in the fields of targeted drug delivery, biodetection, etc. This research provides a versatile method to prepare biodegradable block copolymer bioconjugates.

ASSOCIATED CONTENT

Supporting Information. ^{1}H NMR, GPC elution chromatograms. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: nkliul@yahoo.com (L.L.); hyzhao@nankai.edu.cn (H.Z.).

Author Contributions

§Jie Jin and Dongxia Wu contributed equally to this work.

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