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Chemosynthesis of Poly(ε-lysine)-Analogous Polymers by Microwave-Assisted Click Polymerization

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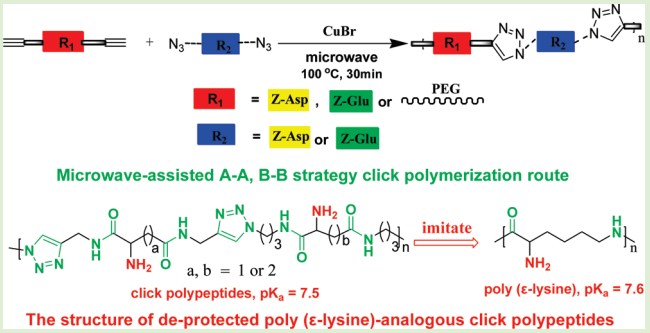
Supporting Information

ABSTRACT: Poly(ε-lysine) (ε-PL)-analogous click polypeptides with not only similar α-amino side groups but also similar main chain to ε-PL were chemically synthesized for the first time through click polymerization from aspartic (or glutamic)-acid-based dialkyne and diazide monomers. With microwave-assisting, the reaction time of click polymerization was compressed into 30 min. The polymers were fully characterized by NMR, ATR-FTIR, and SEC-MALLS analysis. The deprotected click polypeptides had similar pK_a value (7.5) and relatively low cytotoxicity as ε-PL and could be used as substitutes of ε-PL in biomedical applications, especially in endotoxin selective removal. Poly(ethylene glycol) (PEG)-containing alternating copolymers with α-amino groups were also synthesized and characterized. After deprotection, the polymers could be used as functional gene vector with PEG shadowing system and NCA initiator to get amphiphilic graft polymers.



INTRODUCTION

As a kind of unusual cationic, naturally occurring homopoly(amino acid), biosynthesized poly(ε-lysine) (ε-PL) that bears α-amino groups on the side chains is composed of a linkage between the ε-amino groups and carboxyl groups of L-lysine.[1] Compared with chemically synthesized poly(L-lysine) (PLL, composed of linkage between the α-amino groups and carboxyl groups of L-lysine) (pK_a = 9 to 10), ε-PL is edible and nontoxic with lower pK_a (7.6).[1] The existence of α-amino groups in ε-PL seems play the important role of sterilization, antisepsis, gene transfection, and endotoxin removal applications. However, for the difficulties of getting large cyclic monomers (nine-member *N*-carboxyl-ε-amino acid anhydride (ε-NCA) or oenantholactam) of L-lysine as well as the poor solubility of α-amino protecting L-lysine in organic solvents, the chemosynthesis of ε-PL has not been reported. Although polymers baring α-amino side groups were already synthesized from aspartic (or glutamic) acid anhydride and diol through the prepolymerization and subsequent polycondensation,[2−4] the synthesis of anhydride and polycondensation were both water-removal procedures that are difficult to control. The so-formed ester bonds were greatly different from the amide bonds in ε-PL and liable to degrade. Recently, click chemistry,[5] especially copper(I)-catalyzed 1,3-dipolar azide−alkyne cycloaddition (CuAAC)[6−22] attracted our attention. This kind of reaction is tolerable to water, oxygen, and a wide range of functionalities and proceeded with almost quantitative yields under mild conditions in different reaction media. Moreover, the triazole rings resulted by CuAAC could perfectly imitate the functions of amide bonds even including spatial structures for the similar atomic spacing and dipole between the two.[23,24] This interesting discovery makes it possible for chemical synthesis of α-amino side groups containing polymer to imitate ε-PL more similarly through click chemistry.

Until now, there were many attainments on click polymerization to get linear copolymers with triazole units in the main chain,[25] regardless of A−B strategies[26−32] or A−A and B−B strategies.[15−17] Also, there were some reports concerning click chemistry-synthesized polypseudopeptides with amino side groups. However, the amino side groups were mostly ε-amino groups;[29,32] although the starting monomers were based on natural amino acid, they needed to be functionalized by unnatural amino acids, such as N_3−Phe[27,28] and azido-(L)-Lys (Boc),[31,32] which were difficult to synthesize, or the main chains contained ester bonds.[36] Moreover, conventional click polymerizations were time-consuming.[26−30,33−39] Recently, microwave-assisted click chemistry emerged as the time-saving requirement, which could greatly shorten the reaction time of click polymerization.[31,32]

In this work, we first report the design and synthesis of a series of ε-PL-analogous polypeptides and PEG-containing alternating copolymers with α-amino groups on the side chains by the

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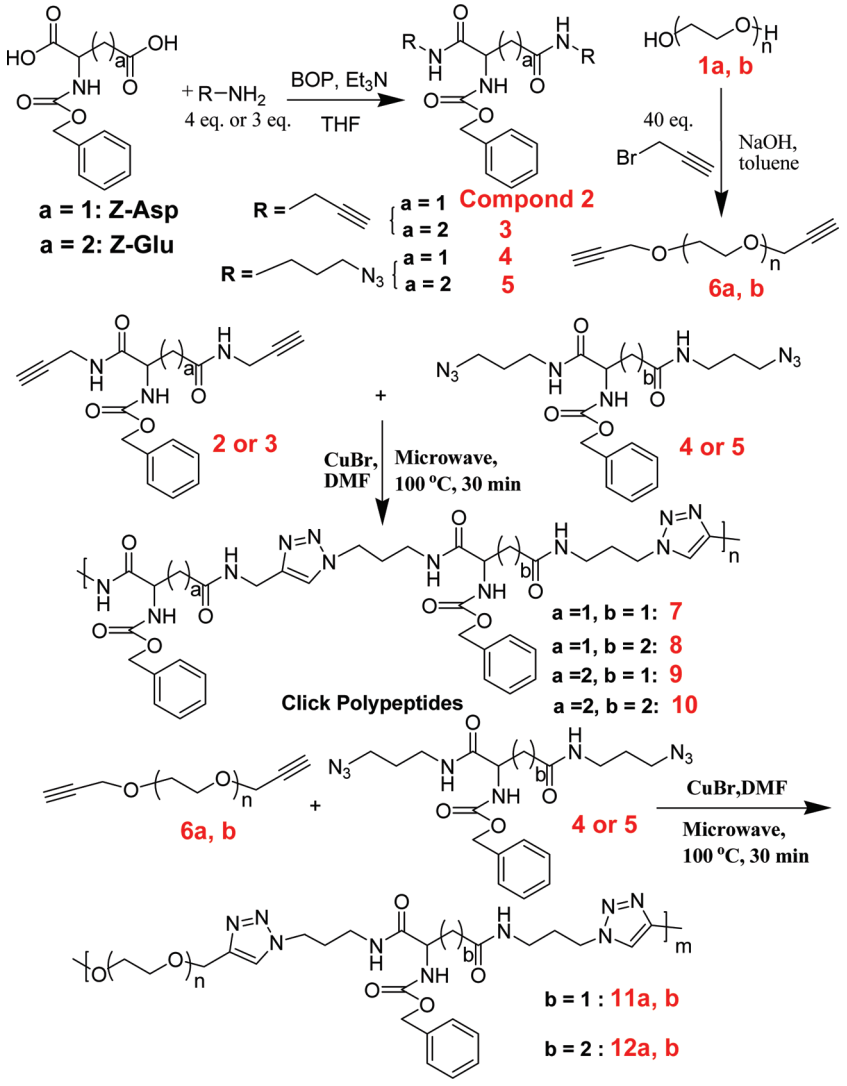
Scheme 1. Click Polymerization of Diazide and Dialkyne Monomers 2−5, 6a, and 6b



combination of the A−A and B−B strategy click polymerization with microwave-assisting technique (Scheme 1).

EXPERIMENTAL SECTION

General. *N*-Benzyloxycarbonylaspartic acid (Z-Asp) and *N*-benzyloxycarbonylglutamic acid (Z-Glu) were synthesized according to traditional peptide synthesis. 3-Azidopropanamine was synthesized by adapting synthetic procedures described previously.[11,40] Poly(ethylene glycol) (PEG) with an average M_n of 950−1050 (**1a**) and 1900−2200 (**1b**), respectively, was purchased from Sigma-Aldrich. ε-Polylysine (ε-PL) was provided by Zhejiang Silver-Elephant bioengineering. Standard endotoxins (ETs) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Tachypleus (or Limulus) amebocyte lysate (TAL or LAL) (with a detection limit of 0.03 EU/mL) and TAL (LAL) reagent water (endotoxin concentration <0.005 EU/mL) were from Zhanjiang Bokang Ocean Creature Company. Bovine serum albumin fraction V (BSA, 99%) was from Sigma-Aldrich. γ-Globulins (from bovine blood, 99%) and cytochrome *c* (from bovine heart, mol wt: 12 327 Da, 95%) were imported from Sigma and packaged by Beijing Solarbio Science & Technology. Hemoglobin (bovine erythrocytes, BR) was imported and packaged by Shanghai Kayon Biological Technology. Propargylamine and propargyl bromide were from Tianzunzezhong chemical limited corporation (Nanjing, China). BOP reagent and trifluoroacetic acid (CF_3COOH) were from GL Biochem (Shanghai, China). Hydrobromic acid in glacial acetic acid (HBr/AcOH, 33%, w/v) was purchased from Alfa Aesar and used without further purification. Copper(I) bromide was washed with glacial acetic acid, followed by washing with methanol and diethyl ether. After drying under vacuum, it was kept under nitrogen atmosphere before use. Tetrahydrofuran (THF) was dried by refluxing with sodium and then distilled after being immersed with CaH_2 for more than 2 weeks. Triethylamine (TEA) was dried by refluxing with CaH_2 and then distilled. All other reagents were commercially available and used without further purification.

The microwave reactions were carried out in an MCR-3 microwave reactor (Yuhua Instruments, Zhengzhou, China). The pocket-sized pH meter used in pK_a determination was from Hanna instruments company (Italy). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE DRX 300 or 400 spectrometer in $CDCl_3$, D_2O, or DMSO-d_6. The MALDI-TOF/TOF mass spectra were recorded on an autoflexIII smart beam mass-spectrometer of Bruker Daltonics. Differential scanning

calorimetry (DSC) and thermal gravimetric analysis (TGA) were taken by Perkin-Elmer at a heating rate of 20 °C/min under a nitrogen atmosphere. We performed size exclusion chromatography−multiangle laser light scattering (SEC-MALLS) analysis by combining a Water-515 GPC equipped with Waters Styragel HMW6E column (eluent: 10 mM LiBr/DMF, flow rate: 1.0 mL/min, 40 °C) and a DAWN EOS MALS detector (Wyatt Technology, laser wavelength: 690.0 nm). Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra were measured with a Bruker Vertex 70 spectrometer. Concentration of endotoxin was assayed by a Limulus test involving turbidimetric time assay at 450 nm with Toxinometer BET-16 (Tianda Tianfa, Tianjin, China) at 37 °C. Protein concentration was determined by absorbencies at 280 nm (proteins other than cytochrome *c*) or 410 (cytochrome *c*) or 406 nm (hemoglobin) using a UV-2450 spectrometer (Shimadzu, Japan) with minimum wavelength resolution of 0.2 nm.

Synthesis of Dialkyne and Diazide Monomers. *Synthesis of N-Benzyloxycarbonylaspartic Dipropargyldiamide (Dipropargyl Z-Asp) (**2**).* Z-Asp (1.33 g, 5 mmol) and dried triethylamine (TEA) (4 mL, 28 mmol) were dissolved in 30 mL of dried THF under a nitrogen atmosphere with stirring. After the addition of propargylamine (1.4 mL, 20 mmol) and BOP reagent (5.30 g, 12 mmol), the reaction mixture was kept stirring under nitrogen atmosphere for 24 h. The solvent was removed under reduced pressure, and the residual was dissolved in DMF. The DMF solution was poured in 1 M $KHSO_4$ aqueous solution. The crude product was filtered and washed completely with water, 5% $NaHCO_3$ aqueous solution, and water and finally dried under vacuum at 40 °C for 24 h to give a white solid (1.55 g, yield 91%). ^{1}H NMR (Figure S1 in Supporting Information) (300 MHz; DMSO-d_6; δ): 2.4 (2H, m, CHC**H**$_2$CO), 3.08 (2H, t, C≡C**H**), 3.83 (4H, br, C**H**$_2$C≡CH), 4.37 (1H, t, C**H**CO), 5.0 (2H, s, C**H**$_2$Ph), 7.35 (5H, br, **Ph**), 7.42, 8.28, 8.39 (3H, m, 3 CON**H**). ^{13}C NMR (Figure S2 in the Supporting Information) (101 MHz; DMSO-d_6; δ): 28.0 (C$\underline{H}_2$C≡CH), 37.2 (CH$\underline{C}$H$_2$CO), 51.4 (C$\underline{H}$CO), 65.5 (C$\underline{H}_2$Ph), 73.0 (C≡C$\underline{H}$), 80.7 (C$\underline{C}$≡CH), 127.7−128.3 and 136.9 (**Ph**), 155.7 (NH$\underline{C}$OO), 168.9 (NH$\underline{C}$OCH), 170.9 (NH$\underline{C}$OCH$_2$). MALDI-TOF/TOF, $C_{18}H_{19}N_3O_4$ $[M+Na]^+$ calcd: 364.1, observed: 364.1; $[M+K]^+$ calcd: 380.2, observed: 380.1.

*Synthesis of N-Benzyloxycarbonylglutamic Dipropargyldiamide (Dipropargyl Z-Glu) (**3**).* Monomer **3** was obtained using Z-Glu and propargylamine according to the same procedure as that for the synthesis of compound **2** with a yield of 90%. ^{1}H NMR (Figure S1 in the Supporting Information) (300 MHz; DMSO-d_6; δ): 1.68−1.84 (2H, d, CHC**H**$_2$CH$_2$CO), 2.09 (2H, t, CHCH$_2$C**H**$_2$CO), 3.00−3.07 (2H, m, C≡C**H**), 3.80 (4H, m, C**H**$_2$C≡CH), 3.93 (1H, t, C**H**CO), 4.97 (2H, s, C**H**$_2$Ph), 7.32 (5H, br, **Ph**), 7.38−7.41, 8.22, 8.33 (3H, m, 3 CON**H**). ^{13}C NMR (Figure S2 in the Supporting Information) (101 MHz; DMSO-d_6; δ): 27.7 (C$\underline{H}_2$CH$_2$CO), 28.0 (C$\underline{H}_2$C≡CH), 31.6 (C$\underline{H}_2$CO), 54.3 (C$\underline{U}$HCO), 65.5 (C**H**$_2$Ph), 72.9 (C≡C**H**), 81.1 (C$\underline{C}$≡CH), 127.7−128.4, 137.0 (**Ph**), 156.0 (NH$\underline{C}$OO), 171.3, 171.4 (NH$\underline{C}$OCH and NH$\underline{C}$OCH$_2$). MALDI-TOF/TOF, $C_{19}H_{21}N_3O_4$ $[M+Na]^+$ calcd: 378.2, observed: 378.1; $[M+K]^+$ calcd: 394.2, observed: 394.1.

*Synthesis of N-Benzyloxycarbonylaspartic Di(3-azidoproyl)diamide (Diazido Z-Asp) (**4**).* Monomer **4** was obtained using Z-Asp and 3-azidopropanamine according to the same procedure as that for the synthesis of compound **2** with a yield of 89%; here 3-azidopropanamine was three-fold excess. ^{1}H NMR (Figure S1 in the Supporting Information) (300 MHz; DMSO-d_6; δ): 1.60 (4H, m, C**H**$_2$CH$_2$N$_3$), 2.33−2.44 (2H, m, CHC**H**$_2$CO), 3.09 (4H, t, C**H**$_2$N$_3$), 3.32 (4H, t, C**H**$_2$CH$_2$CH$_2$N$_3$), 4.30 (1H, t, C**H**CO), 5.01 (2H, s, C**H**$_2$Ph), 7.33 (5H, br, **Ph**), 7.40, 7.84−7.87, 7.92−7.96 (3H, m, 3 CON**H**). ^{13}C NMR (Figure S2 in the Supporting Information) (101 MHz; DMSO-d_6; δ): 28.4 (C$\underline{H}_2$CH$_2$N$_3$), 36.0 (C$\underline{H}_2$NH), 37.9 (C$\underline{H}_2$CO), 48.3 (C$\underline{H}_2$N$_3$), 52.0 (C$\underline{H}$CO), 65.5 (C$\underline{H}_2$Ph), 127.1−128.3, 137.0 (**Ph**), 155.7 (NH$\underline{C}$OO), 169.3 (NH$\underline{C}$OCH), 171.1 (NH$\underline{C}$OCH$_2$). MALDI-TOF/TOF, $C_{18}H_{25}N_9O_4$ $[M+Na]^+$ calcd: 454.2, observed: 154.2; $[M+K]^+$ calcd: 470.3, observed: 470.1.

*Synthesis of N-Benzyloxycarbonylglutamic Di(3-azidopropyl)diamide (Diazido Z-Glu) (**5**).* Monomer **5** was obtained using Z-Glu and 3-azidopropanamine according to the same procedure for the synthesis of compound **2** with a yield of 91%; here 3-azidopropanamine was also three-folds excess. ^{1}H NMR (Figure S1 in the Supporting Information) (300 MHz; DMSO-d_6; δ): 1.61−1.86 (4H+2H, m, C**H**$_2$CH$_2$N$_3$ and CHC**H**$_2$CH$_2$CO), 2.09 (2H, t, CHCH$_2$C**H**$_2$CO), 3.09 (4H, m, C**H**$_2$N$_3$), 3.32 (4H, t, C**H**$_2$CH$_2$CH$_2$N$_3$), 3.90 (1H, t, C**H**CO), 5.01 (2H, s, C**H**$_2$Ph), 7.35 (5H, br, **Ph**), 7.40, 7.83−7.86, 7.93−7.96 (3H, m, 3 CON**H**). ^{13}C NMR (Figure S2 in the Supporting Information) (101 MHz; DMSO-d_6; δ): 27.9 (C$\underline{H}_2$CH$_2$CO), 28.4 (C$\underline{H}_2$CH$_2$N$_3$), 31.8 (C$\underline{H}_2$CO), 35.8 (C$\underline{H}_2$NH), 48.4 (C$\underline{H}_2$N$_3$), 54.6 (C$\underline{H}$CO), 65.4 (C$\underline{H}_2$Ph), 127.7−128.3, 137.0 (**Ph**), 155.9 (NH$\underline{C}$OO), 171.5 (NH$\underline{C}$OCH and NH$\underline{C}$OCH$_2$). MALDI-TOF/TOF, $C_{19}H_{27}N_9O_4$ $[M+Na]^+$ calcd: 468.2, observed: 468.2; $[M+K]^+$ calcd: 484.3, observed: 484.2.

*Synthesis of Dialkyne-Terminated PEG (**6a**, **6b**).* Dialkyne-terminated PEGs (**6a**, **6b**) were synthesized by adapting the synthetic procedure previously described.[41] In general, 1 equiv of PEG (**1a**, **1b**) was reacted with the excess amount of propargyl bromide (40 equiv) and NaOH powder (40 equiv) in toluene for 15 h at 50 °C. The solvent was removed under vacuum, and the residual was dissolved in water. The solution was extracted with dichloromethane twice. After being dried with $MgSO_4$, the final product was obtained by precipitation in diethyl ether with a yield of 84%. NMR date for **6b** (Figure S3 in the Supporting Information): ^{1}H NMR (400 MHz; $CDCl_3$; δ): 2.46 (t, C≡C**H**), 3.65−3.72 (br, C**H**$_2$C**H**$_2$O), 4.20 (d, C**H**$_2$C≡CH). ^{13}C NMR (101 MHz; $CDCl_3$; δ): 58.0 (C$\underline{H}_2$C≡CH), 70.2 (C$\underline{H}_2$C$\underline{H}_2$O), 74.4 (C≡C$\underline{H}$), 77.9 (C$\underline{C}$≡CH). The average molecular weights of **6a** and **6b** were calculated from ^{1}H NMR as 1025 and 2152 g/mol, respectively.

Microwave-Assisted Click Polymerizations. *Synthesis of Click Polypeptide **7**.* The synthesis of click polypeptide was carried out in a microwave reactor using CuBr as catalyst according to literature.[30−32,38,39] In general, a solution of dipropargyl Z-Asp-(compound **2**, 170.5 mg, 0.5 mmol), diazido Z-Asp(compound **4**, 215.5 mg, 0.5 mmol), and CuBr (7 mg, 0.05 equiv) in N_2-purged DMF (1 mL) was placed in the microwave reactor and was irradiated at 100 °C for 30 min under a nitrogen atmosphere. The clear solution was turned into a turbid gel. The gel was dissolved in 1 mL of DMF, and the solution was poured in 0.1 M HCl (50 mL). The precipitant was centrifuged, and washed with 0.1 M HCl and water. After lyophilization, the membrane-like white product **7** was obtained with a yield of 90% (347 mg). ^{1}H NMR (Figure 1) (300 MHz; DMSO-d_6; δ): 1.87−1.89 (4H, br, 2 C**H**$_2$CH$_2$NH), 2.45 (4H, m, CHC**H**$_2$CO), 3.03 (4H, br, triazole-CH$_2$CH$_2$C**H**$_2$NH), 3.95 (2H, br, C**H**CO), 4.30 (8H, br, triazole-C**H**$_2$ (CH$_2$)$_2$ NH and triazole-C**H**$_2$NH), 5.00 (4H, s, C**H**$_2$Ph), 7.31−7.33 (10H, br, **Ph**), 7.45−7.47, 7.92, 8.06 (6H, m, CON**H**), 8.34, 8.43 (2H, d, the **H** in the two triazole). ^{13}C NMR (101 MHz; DMSO-d_6; δ): 29.8 (triazole-CH$_2$C**H**$_2$CH$_2$NH), 35.8 (triazole-(CH$_2$)$_2$C**H**$_2$NH), 37.5, 37.8 (CH$\underline{C}$H$_2$CO), 47.0 (triazole-$\underline{C}$H$_2$NH and triazole-$\underline{C}$H$_2$ (CH$_2$)$_2$ NH), 51.8, 52.0 (C$\underline{H}$CO), 65.5 (C$\underline{H}_2$Ph), 122.9 (C=C$\underline{H}$ on triazole), 127.7, 127.8, 128.3, and 136.9 (**Ph**), 144.7 (C$\underline{C}$=CH on triazole), 155.7 (NH$\underline{C}$OO), 169.2, 169.4, 171.1, and 171.2 (C$\underline{O}$NH on the main chain). ATR-FTIR (cm^{-1}): 3288 (CO$\underline{NH}$), 3064 (triazole), 1711 (OCONH of Z groups), 1657 (CONH in the main chain), 740, 698 (Ph).

*Synthesis of Click Polypeptide **8**.* Click polypeptide **8** was obtained using dipropargyl Z-Asp (compound **2**, 170.5 mg, 0.5 mmol) and diazido Z-Glu (compound **5**, 222.5 mg, 0.5 mmol) according to the same procedure for the synthesis of polypeptide **7** with a yield of 89% (350 mg). ^{1}H NMR (300 MHz; DMSO-d_6; δ): 1.70−1.90 (6H, br, CH$_2$C**H**$_2$CH$_2$NH and C**H**$_2$CH$_2$CO), 2.14 (2H, br, CH$_2$C**H**$_2$CO), 2.45 (2H, m, CHC**H**$_2$CO), 3.04 (4H, br, triazole-(CH$_2$)$_2$ C**H**$_2$NH), 3.93,

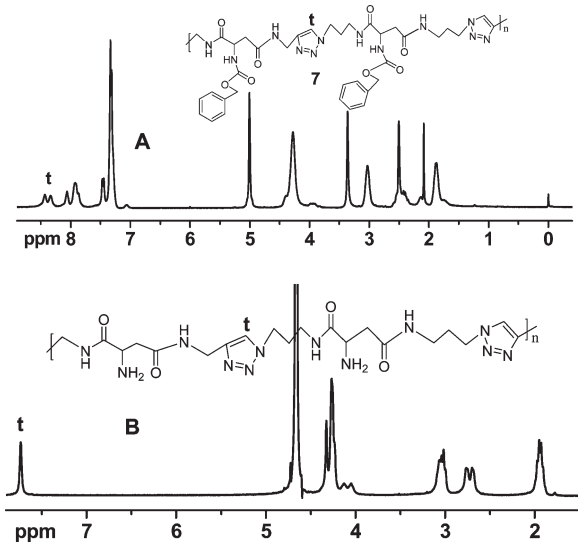


Figure 1. ^{1}H NMR spectra of click polypeptide **7** (A) before and (B) after deprotection.

4.28−4.40 (10H, d, triazole-C**H**$_2$NH, COC**H**CH$_2$CO, COC**H** $(CH_2)_2$ CO and triazole-C**H**$_2$ $(CH_2)_2$NH), 5.01 (4H, s, C**H**$_2$Ph), 7.34 (10H, br, **Ph**), 7.43−7.45, 7.96, 8.06 (6H, m, CON**H**), 8.35, 8.44 (2H, d, the **H** in the two triazole). ^{13}C NMR (101 MHz; DMSO-d_6; δ): 27.8 (**C**H$_2$CH$_2$CO), 29.8 (triazole-CH$_2$**C**H$_2$CH$_2$NH), 31.7 (CH$_2$**C**H$_2$CO), 35.7 (triazole-$(CH_2)_2$**C**H$_2$NH), 37.7 (CH**C**H$_2$CO), 47.0 (triazole-**C**H$_2$NH and triazole-**C**H$_2$ $(CH_2)_2$ NH), 51.9 (**C**HCH$_2$CO), 54.4 (**C**H $(CH_2)_2$CO), 65.5 (**C**H$_2$Ph), 123.2 (C=**C**H on triazole), 127.0, 127.6, 128.2, and 136.9 (**Ph**), 144.9 (**C**=CH on triazole), 155.7, 155.9 (NH**C**OO), 169.4, 171.1, and 171.4 (**C**ONH on the main chain).

*Synthesis of Click Polypeptide **9**.* Click polypeptide **9** was obtained using dipropargyl Z-Glu (compound **3**, 177.5 mg, 0.5 mmol) and diazido Z-Asp (compound **4**, 215.5 mg, 0.5 mmol), according to the same procedure for the synthesis of polypeptide **7** with a yield of 90% (354 mg). ^{1}H NMR (300 MHz; DMSO-d_6; δ): 1.70−1.90 (6H, br, CH$_2$C**H**$_2$CH$_2$NH and C**H**$_2$CH$_2$CO), 2.16 (2H, br, CH$_2$C**H**$_2$CO), 2.40 (2H, m, CHC**H**$_2$CO), 3.04 (4H, br, triazole-$(CH_2)_2$ C**H**$_2$NH), 4.00, 4.30 (10H, d, triazole-C**H**$_2$NH, COC**H** $(CH_2)_2$ CO, triazole-C**H**$_2$ $(CH_2)_2$ NH and COC**H**CH$_2$CO), 5.01 (4H, s, C**H**$_2$Ph), 7.32−7.34 (10H, br, **Ph**), 7.45−7.47, 7.96, 8.07 (6H, m, CON**H**), 8.32, 8.43 (2H, d, the **H** in the two triazole). ^{13}C NMR (101 MHz; DMSO-d_6; δ): 27.9 (**C**H$_2$CH$_2$CO), 29.9 (triazole-CH$_2$**C**H$_2$CH$_2$NH), 31.9 (CH$_2$**C**H$_2$CO), 35.9 (triazole-$(CH_2)_2$**C**H$_2$NH), 37.7 (CH**C**H$_2$CO), 47.3 (triazole-**C**H$_2$NH and triazole-**C**H$_2$ $(CH_2)_2$ NH), 51.9 (**C**HCH$_2$CO), 54.7 (**C**H $(CH_2)_2$CO), 65.6 (**C**H$_2$Ph), 123.2 (C=**C**H on triazole), 127.7, 128.4, and 137.0 (**Ph**), 145.2 (**C**=CH on triazole), 155.9, 156.0 (NH**C**OO), 169.4, 171.2, and 171.8 (**C**ONH on the main chain).

*Synthesis of Click Polypeptide **10**.* Click polypeptide **10** was obtained using dipropargyl Z-Glu (compound **3**, 177.5 mg, 0.5 mmol) and diazido Z-Glu (compound **5**, 222.5 mg, 0.5 mmol), according to the same procedure for the synthesis of polypeptide **7** with a yield of 90% (360 mg). ^{1}H NMR (300 MHz; DMSO-d_6; δ): 1.75−1.92 (8H, br, CH$_2$C**H**$_2$CH$_2$NH and C**H**$_2$CH$_2$CO), 2.14 (4H, br, CH$_2$C**H**$_2$CO), 3.03 (4H, br, triazole-$(CH_2)_2$ C**H**$_2$NH), 3.91−4.00 (2H, m, COC**H** $(CH_2)_2$ CO), 4.30 (8H, br, triazole-C**H**$_2$NH and triazole-C**H**$_2$ $(CH_2)_2$ NH), 5.01 (4H, s, C**H**$_2$Ph), 7.34 (10H, br, **Ph**), 7.44−7.46, 7.90−7.92, 8.05 (6H, m, CON**H**), 8.32, 8.43 (2H, d, the **H** in the two triazole). ^{13}C NMR (101 MHz; DMSO-d_6; δ): 27.9 (**C**H$_2$CH$_2$CO), 29.9 (triazole-CH$_2$**C**H$_2$CH$_2$NH), 31.7 (CH$_2$**C**H$_2$CO), 35.8 (triazole-$(CH_2)_2$-**C**H$_2$NH), 47.0, 47.2 (triazole-**C**H$_2$NH and triazole-**C**H$_2$ $(CH_2)_2$ NH), 54.4, 54.6 (CO**C**H), 65.5 (**C**H$_2$Ph), 122.9 (C=**C**H on triazole), 127.7, 128.3, and 137.0 (**Ph**), 144.7 (**C**=CH on triazole), 155.9 (NH**C**OO), 17.4, 171.6 (**C**ONH on the main chain).

Synthesis of PEG-Containing Alternating Copolymers. Copolymers **11a**, **11b**, **12a**, and **12b** were synthesized using the same procedure. Here the synthesis of **11a** was shown below as an example. Dialkyne-terminated PEG 1000 (**6a**) (216 mg, more than 0.21 mmol), compound **4** (86.2 mg, 0.2 mmol), triethylamine (28 μL, 0.5 equiv), and CuBr (3 mg, 0.05 equiv) were mixed in N_2-purged DMF (2 mL) and settled in the microwave reactor. The reaction mixture was irradiated at 100 °C for 30 min under a nitrogen atmosphere. The solution was poured in diethyl ether (containing a little amount of triethylamine to remove copper ions). The precipitant was centrifuged and washed with diethyl ether. Copolymer **11a** was finally obtained after being dried under vacuum.

Because the structures of **11b** and **12b** were similar to that of **11a** and **12a**, respectively, only the NMR data and spectra of **11a** and **12a** are given below:

NMR date of **11a** (Figure S4 in the Supporting Information): ^{1}H NMR (300 MHz; DMSO-d_6; δ): 1.87−1.89 (4H, br, 2 CH$_2$C**H**$_2$CH$_2$NH), 2.45 (2H, m, CHC**H**$_2$CO), 300−3.02 (4H, br, 2 triazole-$(CH_2)_2$ C**H**$_2$NH), 3.28−3.47 (85.0 H, br, C**H**$_2$C**H**$_2$O of PEG), 4.10 (0.69H, s, C**H**$_2$ that attach to the alkyne terminal of PEG), 4.28 (5H, br, triazole-C**H**$_2$ $(CH_2)_2$ NH and C**H**CO), 4.48 (4H, s, triazole-C**H**$_2$O), 4.98 (2H, s, C**H**$_2$Ph), 7.28 (5H, br, **Ph**), 7.40, 7.90 (3H, m, CON**H**), 8.02 (2H, br, the **H** in the two triazole). ^{13}C NMR (101 MHz; DMSO-d_6; δ): 29.8 (triazole-CH$_2$**C**H$_2$CH$_2$NH), 35.6, 35.8 (triazole-$(CH_2)_2$ **C**H$_2$NH), 37.7 (CH**C**H$_2$CO), 46.9 (triazole-**C**H$_2$ $(CH_2)_2$ NH), 51.9 (**C**HCO), 57.4 (**C**≡CH of the alkyne terminal), 63.5 (triazole-**C**H$_2$O), 65.5 (**C**H$_2$Ph), 68.5, 68.9, 69.7 (**C**H$_2$**C**H$_2$O of PEG), 77.0 (C≡**C**H of the alkyne terminal), 123.9 (C=**C**H on triazole), 127.6, 128.2 136.8 (**Ph**), 143.8 (**C**=CH on triazole), 155.6 (NH**C**OO), 1693, 171.1 (**C**ONH on the main chain).

NMR date of **12a** (Figure S5 in the Supporting Information): ^{1}H NMR (300 MHz; DMSO-d_6; δ): 1.69−1.90 (6H, br, CH$_2$C**H**$_2$CH$_2$NH and C**H**$_2$CH$_2$CO), 2.10 (2H, br, CH$_2$C**H**$_2$CO), 3.01 (4H, br, triazole-$(CH_2)_2$ C**H**$_2$NH), 3.29−3.50 (85.0 H, br, C**H**$_2$C**H**$_2$O of PEG), 3.90 (1H, br, C**H**CO), 4.10 (0.46H, d, C**H**$_2$ that attach to the alkyne terminal of PEG), 4.30 (4H, t, triazole-C**H**$_2$ $(CH_2)_2$ NH), 4.47 (4H, s, triazole-C**H**$_2$O), 4.98 (2H, s, C**H**$_2$Ph), 7.25−7.30 (5H, br, **Ph**), 7.39, 7.41, 7.88 (3H, m, CON**H**), 8.04 (2H, br, the **H** in the two triazole). ^{13}C NMR (101 MHz; DMSO-d_6; δ): 27.7 (**C**H$_2$CH$_2$CO), 29.8 (triazole-CH$_2$**C**H$_2$CH$_2$NH), 31.7 (CH$_2$**C**H$_2$CO), 35.7 (triazole-$(CH_2)_2$ **C**H$_2$-NH), 46.9, 47.1 (triazole-**C**H$_2$ $(CH_2)_2$ NH), 54.5 (**C**HCO), 63.5 (triazole-**C**H$_2$O), 65.4 (**C**H$_2$Ph), 68.5, 68.9, 69.7 (**C**H$_2$**C**H$_2$O of PEG), 123.9 (C=**C**H on triazole), 127.6, 128.3, 136.9 (**Ph**), 143.9 (**C**=CH on triazole), 155.9 (NH**C**OO), 171.5 (**C**ONH on the main chain).

Deprotection of Click Polypeptide. As an example, the click polypeptide **7** (400 mg, containing 1 mmol Z groups) was dissolved in CF_3COOH (6 mL) with the addition of 33% HBr/AcOH (4 mL, about 10 equiv to Z groups). The mixture was stirred at 0 °C under a nitrogen atmospheres for 2 h. After that, the solution was concentrated and poured in diethyl ether. The precipitant was dissolved in water and dialyzed against water using a dialysis tube with MW cutoff of 3500 Da for 3 days and then freeze-dried. Deprotected click polypeptide **7** (DCP7) was obtained with a yield of 85% (227 mg). ^{1}H NMR (300 MHz; D_2O; δ): 1.91−1.97 (4H, m, CH$_2$C**H**$_2$CH$_2$NH), 2.70, 2.76 (4H, d, CHC**H**$_2$CO), 3.01−3.04 (4H, br, triazole-$(CH_2)_2$ C**H**$_2$NH), 4.05, 4.13 (2H, d, COC**H**), 4.24−4.33 (8H, br, triazole-C**H**$_2$NH and triazole-C**H**$_2$ $(CH_2)_2$ NH), 7.74 (2H, s, triazole). ^{13}C NMR (Figure S6 in the Supporting Information) (101 MHz; D_2O; δ): 28.3 (triazole-CH$_2$**C**H$_2$CH$_2$NH), 34.0, 34.2 (CH**C**H$_2$CO), 35.8, 36.0 (triazole-$(CH_2)_2$**C**H$_2$NH), 47.3, 47.4 (triazole-**C**H$_2$NH and triazole-**C**H$_2$ $(CH_2)_2$ NH), 49.9 (CO**C**H), 123.4 (**C**=CH on triazole), 143.8 (**C**=CH on triazole), 170.0 (**C**ONH on the main chain). ATR-FTIR (cm^{-1}): 3244 (NH_2), 3071 (triazole), 1657 (CONH in the main chain).

Synthesis of Thermal DCP7. To investigate the effect of Cu ions residual, thermal DCP7 was synthesized through thermal click polymerization, followed by deprotection procedure. The synthesis of thermal click polypeptide **7** was carried out in a microwave reactor but without CuBr catalyst. In general, a solution of dipropargyl Z-Asp(compound **2**, 170.5 mg, 0.5 mmol) and diazido Z-Asp(compound **4**, 215.5 mg, 0.5 mmol) in N_2-purged DMF (1 mL) was placed in the microwave reactor and was irradiated at 120 °C for 75 min under a nitrogen atmosphere. The postprocessing and following deprotection procedure were the same as click polypeptide **7**. ^{1}H NMR (300 MHz; D_2O; δ): 1.58−1.65 and 1.88−1.97 (4H, d, $CH_2CH_2CH_2NH$), 2.70, 2.80 (4H, br, $CHCH_2CO$), 3.04−3.4 (4H, br, triazole-$(CH_2)_2$ CH_2NH), 3.76, 3.83 (br, 2*x*H, 1, 4-triazole-CH_2NH), 4.05, 4.13 (2H, d, $COCH$), 4.24−4.33 ([8−2*x*]H, br, 1, 5-triazole-CH_2NH and triazole-CH_2 $(CH_2)_2$ NH), 7.43 (2xH, s, 1,4-triazole), 7.77 ([2−2*x*]H, s, triazole).

pK_a Determination of Deprotected Click Polypeptide 7. The pK_a of deprotected click polypeptide 7 (DCP7) was determined by potentiometric titration. In general, DCP7 (13.4 mg, with 50 mmol amino groups) was dissolved in distilled water (10 mL) using a beaker equipped with a pH meter. Then, NaOH solution (2.5 mM) was dropwisely added to the solution, and the pH values of the solution accompanied by the addition volume of NaOH solution were recorded and analyzed according to the method below (eq 1).

$$[-NH_3^+] \rightleftarrows [H^+] + [-NH_2]$$

$$K_a = \frac{[H^+][-NH_2]}{[-NH_3^+]}$$

When

$$V = \frac{1}{2} V_a,\ [-NH_2] = [-NH_3^+]$$

So

$$K_a = ([H^+])_{1/2V_a},\ pK_a = (pH)_{1/2V_a} \quad (1)$$

Here $[-NH_3^+]$ and $[-NH_2]$ stand for concentration of the amino groups in ionic state and molecular state.

V_a stands for the volume of the added NaOH solution at the end point of titration, which was determined by the pH−V curve as well as the d(pH)/dV−V curve (the maximum in the d(pH) /dV−V curve) described in Figure S6 of the Supporting Information.

Cytotoxicity of Deprotected Click Polypeptide 7. The relative cytotoxicity of DCP7 was assessed with a methyl tetrazolium (MTT) assay against L929 mouse fibroblast. Biosynthesized ε-PL and chemically synthesized PLL were used as the positive and negative controls, respectively. Because DCP7 could have the possibility of containing a trace amount of the copper ion residual, which would poison cells, microwave-assisted thermal click polymerizations (120 °C, 75 min) without Cu (I) catalyst were also taken to obtain the deprotected click polypeptide 7 without Cu ion (thermal DCP7) for the cytotoxicity assessment. The weighed dry samples (ε-PL, DCP7, thermal DCP7, and PLL) were sterilized by ultraviolet (UV) and then dissolved in RPMI 1640 culture medium. Subsequently, 100 μL of L929 cells in RPMI 1640 culture medium at a density of 5000 cells per well was added to each well in a 96-well plate. Cells were incubated for 48 h in an incubator (37 °C, 5% CO_2), followed by the addition of the sample-containing culture medium to make the final sample concentrations of 1, 0.5, and 0.25 mg mL^{-1}, respectively. After another 24 and 48 h of incubation, the cells were observed using an inverted microscope (Nikon-2000U). We added 20 μL of MTT stock solution (5 mg mL^{-1} in PBS) to each well of the plate for an additional 4 h of incubation. The purple formazan produced by active mitochondria was solubilized using 200 μL of DMSO. The optical density (OD) at 492 nm in each well was measured on a microplate reader. Each sample was measured at least six times, and the average results are shown in Figures 3 and 4.

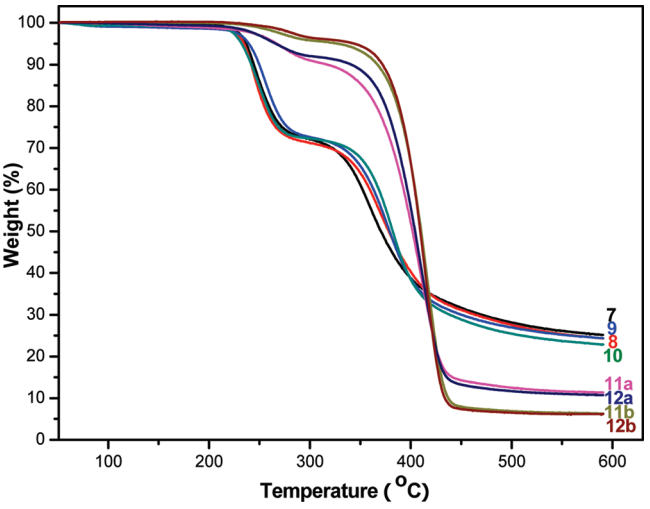


Figure 2. TGA curves of polymers **7**−**10**, **11a**, **11b**, **12a**, and **12b** in N_2.

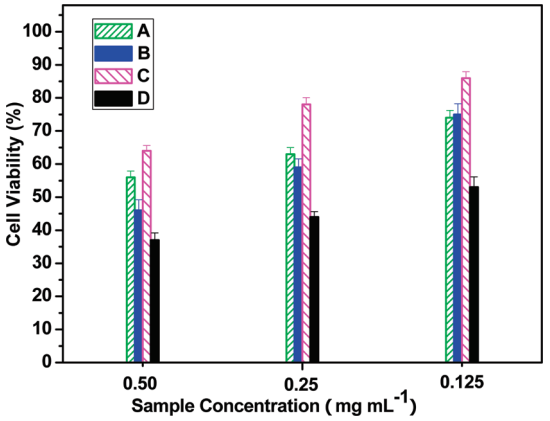


Figure 3. Cytotoxicity study of (A) ε-PL, (B) DCP7, (C) thermal DCP7, and (D) PLL at different polymer concentrations against L929 fibroblasts evaluated by MTT assay after 48 h.

Endotoxin Selective Removal Properties of DCP7. To assess the endotoxin-selective removal properties of DCP7, epoxy-containing cross-linked polystyrene (PS-EO) microspheres was prepared by dispersion polymerization of epoxypropyl methacrylate (EM), styrene (S), and divinyl benzene (DVB). (See the Supporting Information and Scheme S1.) DCP7, ε-PL (as positive control), and PLL (as negative control) were then immobilized to PS-EO microspheres through the reaction between amino groups and epoxy groups. (See the Supporting Information and Scheme S2.) The amino-group contents of DCP7-, ε-PL-, or PLL-immobilized PS-EO microspheres were determined by ninhydrin technique according to literature[42] to be 17.5, 13.0, and 14.5 μmol/g, respectively. The endotoxin adsorption and protein recovery properties of the DCP7-, ε-PL-, or PLL immobilized microspheres with about equal total amino content (DCP7-immobilized PS-EO microspheres: 17.5 μmol/g × 10 mg; ε-PL-immobilized PS-EO microspheres: 13.0 μmol/g × 11 mg; PLL-immobilized PS-EO microspheres: 14.5 μmol/g × 11 mg) were investigated independently. The endotoxin adsorption process was described below: typically, the adsorbent was first treated with 0.2 M NaOH solution, normal saline, and ethanol to remove the original pyrogen and other impurities. The adsorbent was then dispersed by ultrasonic in 1 mL of endotoxin

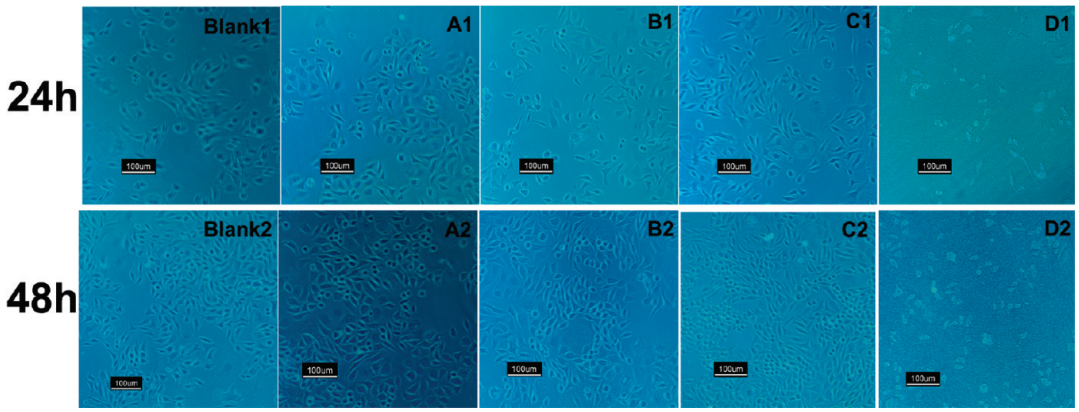


Figure 4. Optical images of the incubated L929 fibroblast cells. **Blank1**, **Blank2**: without polymer (blank); **A1**, **A2**: in 0.5 mg mL^{-1} of ε-PL; **B1**, **B2**: in 0.5 mg mL^{-1} of DCP7; **C1**, **C2**: in 0.5 mg mL^{-1} of thermal DCP7; and **D1**, **D2**: in 0.25 mg mL^{-1} of PLL for 24 h (**Blank1**, **A1**, **B1**, **C1**, and **D1**) and 48 h (**Blank2**, **A2**, **B2**, **C2**, and **D2**).

solution in TAL (LAL) reagent water with a concentration of 10 EU/mL. The mixture was oscillated at 25 °C for 15 min. The adsorbent was centrifuged, and the endotoxin concentration of the supernatant was measured by a Limulus test. The protein recoveries of different polycation (ε-PL, DCP7, or PLL) immobilized PS-EO microspheres were determined as below: after pretreatment, as described in the endotoxin adsorption process, the adsorbent was mixed with 1 mL of protein solution in distilled water. After oscillating at 25 °C for 15 min, the adsorbent was centrifuged, and the protein concentration of the supernatant was determined by UV measurement. As an example, the endotoxin selective removal properties from hemoglobin solution of different polycation (ε-PL, DCP7, or PLL)-immobilized PS-EO microspheres were determined as below: hemoglobin solution that contained ~4500 EU/mL endotoxin was prepared; then, the pretreated adsorbent was mixed with 1 mL of the above-mentioned solution. After oscillating at 25 °C for 15 min, the adsorbent was centrifuged. The endotoxin and hemoglobin concentration of the supernatant were measured. All endotoxin adsorption and protein recovery experiments were repeated three times, and the results were averaged.

RESULTS AND DISCUSSION

Biosynthesized ε-PL has a lot of advantages over chemically synthesized PLL, especially in the application of selective removal of endotoxin from protein solution. Endotoxin (lipopolysaccharide (LPS)), is the constituent of cell walls of Gram-negative bacteria, which largely exists in bioproducts, such as peptides and proteins. The removal of endotoxin even in nanogram quantities from drugs and fluids before injection is critical because of its potent biological activities causing pyrogeneic and shock reactions in mammals. Endotoxin is an amphipathic molecule that has both an anionic region and a hydrophobic region. The removal of endotoxin mainly takes use of its amphipathic properties. In the case of removing endotoxin from protein solution, the adsorption of protein is commonly unavoidable. It seems that the low pK_a (7.6) of ε-PL makes it possible to decrease the interaction with protein, especially the acidic protein like BSA.[43−45] However, the large scale production of ε-PL using biosynthesis technique is inconvenient. The purification procedures are complicated, and the molecular weight of ε-PL is uneasy to control. As chemists, we have been seeking a route to synthesize chemically ε-PL or its substitutes for many years. To synthesize ε-PL straightly from α-amino protected L-lysine or its large cyclic monomers would have many difficulties. To mimic the structure of ε-PL, we must obtain a kind of polymers with not only α-amino side groups but also similar main chain structure to ε-PL. Because of the interesting feature of the triazole groups that it can imitate the character of amide bonds in some cases, click chemistry was chosen as a useful tool to construct the ε-PL-analogous polymers.

Monomer Synthesis. To apply the click polymerization strategy, we should obtain that kind of monomers with diazide or dialkyne groups on both ends of the molecular structures. α-Amino side group is another necessary structural factor. For these reasons, aspartic acid and glutamic acid, which have one α-amino group and two carboxyl groups, were selected as starting reactants, and the amino groups of them were protected by benzyloxycarbonyl groups to give *N*-benzyloxycarbonylaspartic acid (Z-Asp) and *N*-benzyloxycarbonylglutamic acid (Z-Glu). The diazide and dialkyne monomers **2**−**5** were synthesized by amide condensation of Z-Asp and Z-Glu with 3-azidopropanamine or propargylamine in anhydrous THF in the presence of BOP reagent and triethylamine with high yields (~90%). Highly purified monomers could be obtained conveniently because the main byproduct of this reaction was a kind of salt, which could be conveniently washed away with water. Dialkyne-terminated PEG **6a**, **6b** with different molecular weights was also prepared by the reaction of PEG (molecular weight of **1a** and **1b** were 1000 and 2000 Da, respectively) and large excess of propargyl bromide (40 fold) using NaOH as catalyst. All diazide and dialkyne monomers, including **2**−**5** and **6a**, **6b**, were characterized by ^{1}H and ^{13}C NMR (Figures S1−S3 in Supporting Information), indicating high purity. Monomers **2**−**5** were also analyzed by mass spectroscopy.

Click Polypeptides. The click polypeptides were synthesized through click polymerizations of dialkyne **2** or **3** and diazide **4** or **5** monomers. The equal equivalence of purified dialkyne **2** or **3** and diazide **4** or **5** monomers was mixed together in DMF under a nitrogen atmosphere with CuBr as catalyst (0.05 equiv according to alkyne or azide groups). Polymerizations could be successfully processed and finished within 30 min in a microwave reactor. The obtained click polypeptides **7**−**10** were characterized by ^{1}H and ^{13}C NMR as well as SEC-MALLS analyses.

As an example, ^{1}H NMR spectra of click polypeptide **7** is presented in Figure 1. The polyaddition reactions between dialkyne and diazide monomers were confirmed by the appearance of the characteristic signal of triazole rings at 8.34 and 8.43

Table 1. Characteristics of Click Peptide 7-10 and PEG-Containing Multi-Block Copolymers11a,11b and 12a,12b

sample	solubility[a]	M_n (10^4 Da)[b]	PDI[b]	T_g (°C)[c]	T_m (°C)[c]	T_d (°C)[d]
7	**F, S**	2.85	3.32	106.7		236.9
8	**F, S**	2.26	4.03	103.2		232.3
9	**F, S**	3.55	2.30	99.2		239.5
10	**F, S**	5.30	2.34	96.8		229.7
11a	**F, S, W**	6.69	4.12		27.5	267.2
11b	**F, S, W**	3.60	3.35		41.0	328.0
12a	**F, S, W**	9.35	4.11		28.8	266.2
12b	**F, S, W**	2.85	3.86		41.0	344.1

[a] **F** = DMF, **S** = DMSO, **W** = water. [b] By SEC-MALLS analyses in DMF. [c] By DSC (second heating scan) in nitrogen atmospheres. [d] Thermal decomposition temperature (T_d) was recorded by TGA at a temperature of 5.0% decomposition.

ppm in ^{1}H NMR (Figure 1A, t) as well as 122.9 and 144.7 ppm in ^{13}C NMR spectra (Supporting Information Figure S6A). It is noteworthy that because the click polymerization was taken place under high monomer concentration with microwave-assisting, the reaction time was compressed within 30 min,[31,32] and the molecular weights of click polypeptides **7**−**10** were all about several ten of thousands of Da with polydispersities from 2.3 to 4.0, which was determined by SEC-MALLS analyses. (See Table 1 and Supporting Information Figure S7.)

All click polypeptides **7**−**10** were amorphous polymers with high T_g values (∼100 °C) (Table 1). These T_g values were not only much higher than that of poly (γ-benzyl-L-glutamate) (PBLG) (∼19 °C),[10] but also higher than that of nylon66 (45.0 °C), nylon610 (50.0 °C), and even nylon46 (75.0 °C). It is obvious that the rigid triazole structure in the polymer main chain plays the important role of the increase in T_g, as previous reports revealed.[27,35,36] The TGA analyses (Figure 2 and Table 1) of click polypeptides **7**−**10** showed that all click polypeptides were relatively stable with the thermal decomposition temperatures (T_d values) at ∼230 °C. About 30% weight loss occurred from 230 to 270 °C, which was caused by the thermal decomposition of the amino protecting groups of click polypeptide. After that, each TGA curve presented a platform from 270 to 350 °C. Finally, the main chain of the polymers started to break and decompose by the elevation of the temperature to >350 °C. All of the click polypeptides **7**−**10** indicated good solubility in strong polar solvents such as DMSO and DMF.

PEG-Peptide Click Copolymers. To confirm further the reliability of the A−A, B−B strategy click polymerization with microwave technique, we synthesized the PEG-containing alternating copolymers **11a**, **11b**, **12a**, and **12b** by the click polyaddition of dialkyne-terminated PEG **6a** (M_w, 1000 Da) or **6b** (M_w, 2000 Da) and diazide monomer **4** or **5**. Unlike amino acid monomers, dialkyne-terminated PEG did not have one identical molecular weight, which made it difficult to obtain the exactly equivalent molar ratio between the dialkyne-terminated PEG and the diazide amino acid. To avoid the confused results of the final copolymers, we designed the molar quantity of the dialkyne-terminated PEG **6a** or **6b** to be a little excess to that of the diazide monomer **4** or **5** to apply the alkyne terminals to both ends of the PEG-containing multiblock copolymers, which was confirmed by ^{1}H and ^{13}C NMR (Figures S4 and S5 of the Supporting Information).

All of these PEG-containing alternating copolymers showed good solubility in DMSO, DMF, and water. They were semicrystalline polymers and had similar thermal decomposition behavior as that of the click polypeptides **7**−**10** (Figure 2 and Table 1). Replacement of the starting monomers from diazido Z-Asp to diazido Z-Glu showed little effect on the T_m values of the copolymers. However, the T_m values of the copolymers were much lower than that of the normal PEG polymer with similar molecular weight (T_m of PEG 20000 is 61 °C). We deduced that the existence of the triazole groups and the huge protecting groups hindered the ordered arrangement of the small PEG chains, resulting in the decrease in the crystallinity. It was interesting that when PEG 2000 was used instead of PEG 1000, the copolymers with lower molecular weight would be obtained (**11b** and **12b**). Extended reaction time to 75 min would have little effect on the molecular weight. We supposed that the longer chains of PEG 2000 would provide the more serious shielding circumstance, making it more difficult for the alkyne groups on PEG to encounter with the diazido monomers during the same reaction time.

***ε*-PL-Analogous Click Polypeptides.** When the above synthesized Z-protected click polypeptides were treated with CF_3COOH/HBr for 2 h at 0 °C under a nitrogen atmosphere, the ε-PL-analogous polypeptides with abundant and regularly arranged α-amino groups on the side chains could be obtained successfully, as illustrated in Scheme 2. As an example, after the deprotection procedure, the signals in ^{1}H and ^{13}C NMR of Z groups on polypeptide **7** disappeared completely, the signals of triazole groups still remained (Figure 1B, t, and Figure S6B of the Supporting Information), and the infrared adsorption of benzyl groups in 740 and 698 cm^{-1} vanished, too (Figure S8 of the Supporting Information). These results clearly indicated the complete deprotection of click polypeptides with the main chains keeping invariant under our treating conditions.

The main properties of the deprotected click polypeptides, including pK_a value and cell cytotoxicity, were investigated and compared with that of the biosynthesized ε-PL from literatures reported to evaluate whether the chemically synthesized click polypeptides possess the similar biofunction as ε-PL. The pK_a of DCP7 determined by conventional potentiometric titration was ∼7.5, which was very close to that of ε-PL (pK_a = 7.6) (Figure S9 of the Supporting Information). The results showed that the α-amino groups on the DCP7 have the similar dissociation property as ε-PL. Unlike the normal PLL, which bared strong cationic character (pK_a = 9 to 10), the lower values of pK_a of the click polypeptides would be very important for the selective removal of the endotoxin from a protein containing biological reagent because many acidic proteins possess negative charge under physiological conditions.

As a kind of cationic polymer, DCP7 could also have some toxicity because the cationic property would have the possibility to destroy the cell membrane. The cytotoxicity of DCP7 was evaluated against L929 mouse fibroblasts in vitro by MTT assay. Chemically synthesized PLL and biosynthesized ε-PL were used as the negative and positive controls, respectively. As shown in Figure 3, the DCP7 exhibited close cytotoxicity to that of ε-PL but had much lower cytotoxicity than that of PLL in all three different concentrations. However, considering the negative effect to the cytotoxicity results, which might come from the harmful copper ions remaining in DCP7 even in trace amount, we also synthesized another DCP sample by microwave-assisted thermal click polymerizations (120 °C, 75 min) without Cu (I) catalyst. (The sample is called thermal DCP7 for short.) This time, thermal DCP7 showed much better cell compatibility than

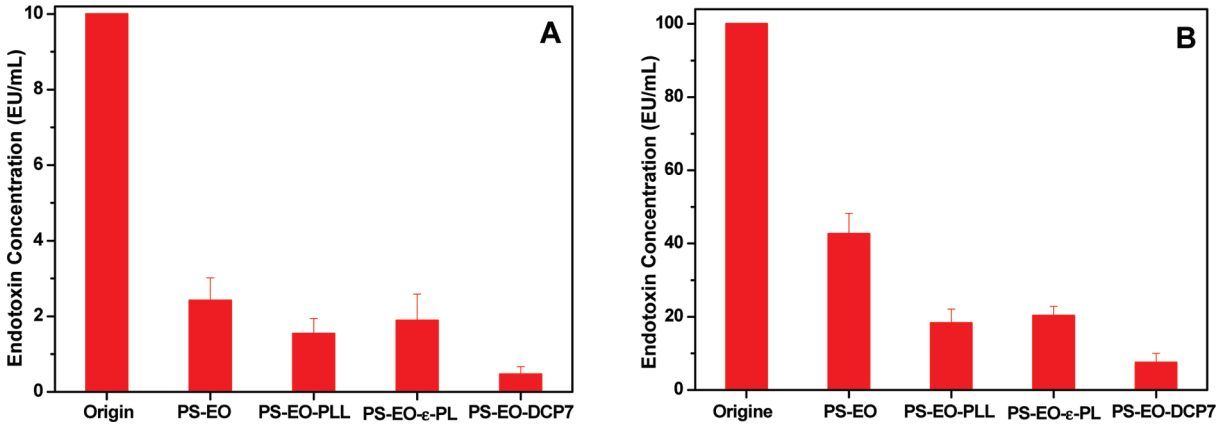
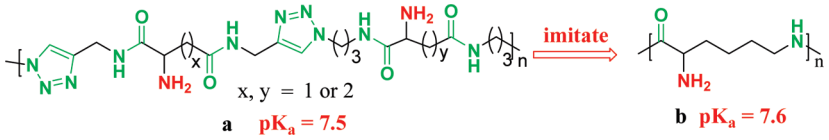


Figure 5. Change of endotoxin concentration before (Origin) and after treatment with polycation-immobilized epoxy-containing cross-linked polystyrene (PS-EO) microspheres. We treated 1 mL of endotoxin solution (10 EU/mL or 100 EU/mL) with about equal total amino content of polycation immobilized microspheres (DCP7- immobilized PS-EO microspheres: 17.5 μmol/g × 10 mg; ε-PL-immobilized PS-EO microspheres: 13.0 μmol/g × 11 mg; PLL-immobilized PS-EO microspheres: 14.5 μmol/g × 11 mg).

Scheme 2. Structure Imitation of Poly(ε-lysine) (b) with De-Protected Click Polypeptides (a)



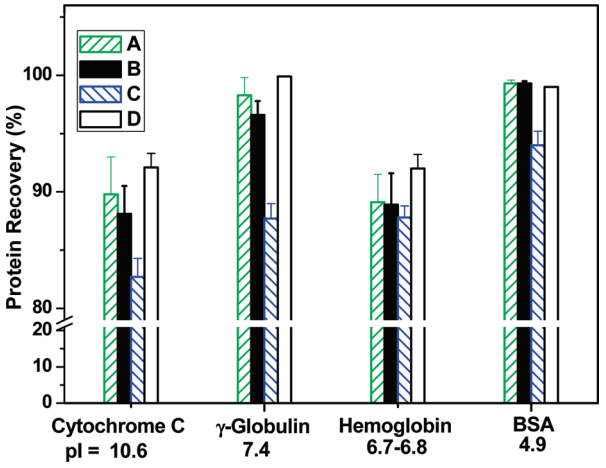


Figure 6. Protein recoveries after treatment with different polycations immobilized epoxy-containing cross-linked polystyrene (PS-EO) microspheres. (A) PS-EO-ε-PL, (B) PS-EO−DCP7, (C) PS-EO-PLL, and (D) PS-EO. The concentrations of cytochrome *c*, γ-globulin, hemoglobin, and BSA were 0.278, 0.456, 0.496, and 0.49 mg/mL, respectively.

ε-PL (Figure 3). Because there are only little difference in the main chain structures between DCP7 and thermal DCP7 (the triazole groups in DCP7 are all 1,4-triazole structures, whereas thermal DCP7 has a little amount of 1,5-triazole structure), the cytotoxicity value of thermal DCP7 could be considered to be the real index of DCP7 without any interference from the copper ions. More intuitional results could be obtained from the optical images of L929 fibroblast cells after incubation for 24 and 48 h (Figure 4). In the PLL (**D1**, **D2**) system, most of the cells died within 24 h, and the remaining living cells were smaller than those other systems even in the lower PLL concentration (0.25 mg mL^{-1}). At the same time, the cells incubated in the solution of 0.5 mg mL^{-1} DCP7 (Figure 4; **B1**, **B2**) as well as thermal DCP7 (**C1**, **C2**) showed good growing status during 48 h, which was quite similar to that of ε-PL (**A1**, **A2**) and blank (**Blank1**, **Blank2**), indicating the high compatibilities of the polymer systems.

As an application example, DCP7, ε-PL (as positive control) and PLL (as negative control) were immobilized onto the epoxy-containing cross-linked polystyrene (PS-EO) microspheres (see Figures S10 and S11 of the Supporting Information for the characterization of PS-EO microspheres) for the evaluation of the endotoxin-selective removal properties. The results showed that after treatment with the polycation (DCP7, ε-PL, or PLL)-immobilized microspheres, the endotoxin concentration remained in the solution decreased sharply from the initial 10 EU/mL to <2 EU/mL (Figure 5A). The PS-EO microspheres themselves also showed some endotoxin absorbing capacity for its hydrophobic properties. It is noteworthy that DCP7-immobilized microspheres exhibited the highest endotoxin removal ability. More than 96% of the endotoxin was removed by the DCP7-immobilized microspheres with an endotoxin adsorption capacity of 953 EU/g. When the initial concentration of endotoxin increased to 100 EU/mL, the endotoxin adsorption capacity of all of the adsorbents increased, but the difference between them enlarged (Figure 5B). At this time, the endotoxin adsorption capacity of DCP7 increased to 9250 EU/g. It has been mentioned that the hydrophobic adsorbent can only adsorb the single endotoxin chain through hydrophobic interaction however, the cationic adsorbent can also adsorb the supramolecular aggregates (micelle or vesicles) formed by endotoxin molecules that have anionic groups on their surface.

Protein recovery is another important character for the endotoxin-adsorbing material because protein is the most

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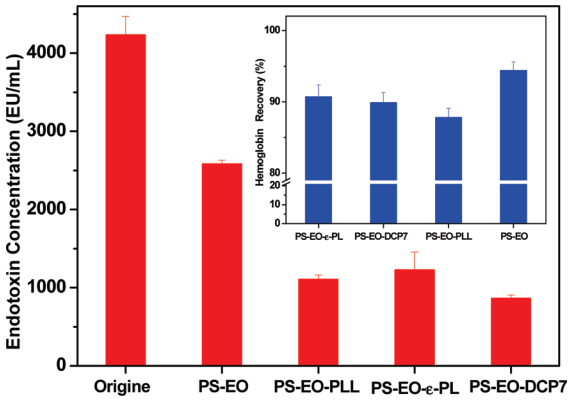

Figure 7. Endotoxin adsorption and hemoglobin recovery properties of different polycations immobilized epoxy-containing cross-linked polystyrene (PS-EO) microspheres. The concentrations of hemoglobin and endotoxin were 0.721 mg/mL and 4230 EU/mL, respectively.

commonly involved component during the actual endotoxin removal process, like endotoxin removal from bacterial-possessed protein solution and blood. To determine the protein recovery, we mixed the polycation (ε-PL, DCP7, or PLL)-immobilized PS-EO microspheres with different protein solutions and checked the changes of the protein concentrations. As shown in Figure 6, without polycations immobilization, the blank PS-EO microspheres (D) showed little effect on the protein concentrations with high protein recoveries (all higher than 90%). After immobilized with polycations, the protein recoveries of the microspheres decreased in all protein solutions except for BSA. Among them, PLL- immobilized microsphere (C) had the lowest protein recoveries, whereas DCP7-immobilized microsphere (B) had significantly higher protein recoveries that were very close to that of the blank microspheres and ε-PL-immobilized microsphere (**A**), indicating the excellent low protein adsorption property for its low pK_a. It is noteworthy that the protein adsorption behaviors of the polycations-immobilized microspheres were not coincident with the protein charges. For example, as the acidic protein with a pI (isoelectric point) of 4.9, BSA should be mostly adsorbed by the polycation immobilized microspheres, theoretically. However, the results showed that the BSA recoveries of the three kinds of polycation-immobilized microspheres were all the highest compared with other proteins. The abnormal phenomenon is assumed to be caused by the low amino contents of the adsorbents and the surface property of cross-linked PS-EO microspheres.

To confirm further the selective removal properties of DCP7 immobilized microspheres, the removal of endotoxin from a endotoxin mixed protein solution was investigated using hemoglobin as an example. As shown in Figure 7, after being treated by polycation (DCP7, ε-PL, or PLL)-immobilized microspheres, the endotoxin concentration that remained in the mixture solution decreased vastly from the initial 4230 to ~1000 EU/mL (Figure 7). Again, DCP7 exhibited the best adsorption ability with an endotoxin adsorption capacity of 3.4×10^5 EU/g. At the same time, >90% of the hemoglobin still remained. The protein recovery results of all of the adsorbents remained consistent with the results obtained by protein recovery experiments from pure hemoglobin solution (Figure 7, top right corner). It is obvious that trace amount of the endotoxin in the protein solution could be removed easily and completely without much loss of the protein content.

The characteristic results above bring the great possibility for the chemosynthesized deprotected click polypeptides (DCPs) to imitate ε-PL in its main properties and to be applied in the endotoxin selective removal area.

CONCLUSIONS

We developed a novel strategy for the elaboration of click polypeptides and PEG-containing alternating copolymers with triazole units in the main chain and potential amino groups on the side chains by microwave-assisted click polymerizations using aspartic (or glutamic)-acid based dialkyne and diazide monomers. It is really an encouragement that the combination of the A−A, B−B strategy click polymerization with microwave technique could provide us a convenient way to synthesize copolymers baring α-amino groups on the side chains with regular arrangement. It is not only a chemically available approach to synthesize the ε-PL-analogous polymers but also the new designation to cultivate the new series of the cationic polymers, which would have the great potential to be used as functional gene vector with PEG as shadowing system as well as NCA initiator to get amphiphilic graft polymers.

ASSOCIATED CONTENT

Supporting Information. ^{1}H and ^{13}C NMR spectra of compounds **2**−**5**, **6a**, **11a**, and **12a**, the ^{13}C NMR and ATR-FTIR spectra of click polypeptide **7** before and after deprotection, the pH−V curve and d(pH)/dV−V curve of deprotected click polypeptide **7**, SEC-MALLS curves of click polypeptides **7**−**10** and PEG-containing alternate copolymers **11a**, **11b** and **12a**, **12b**, the preparation of epoxy-containing cross-linked polystyrene (PS-EO) microspheres, and the immobilization of polycations processes, the SEM and XPS characterizations of cross-linked PS-EO microspheres. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Shih, I.-L.; Shen, M.-H.; Van, Y.-T. *Bioresour. Technol.* **2006**, *97*, 1148–1159.

(2) Won, C.-Y.; Chu, C.-C.; Lee, J. D. *J. Polym. Sci. Part A: Polym. Chem. Ed.* **1998**, *36*, 2949–2959.

(3) Zhao, J.; Quan, D. P.; Liao, K. R.; Wu, Q. *Macromol. Biosci.* **2005**, *5*, 636–643.

(4) Xie, Z.; Lu, C.; Chen, X.; Chen, L.; Hu, X.; Shi, Q.; Jing, X. *Eur. Polym. J.* **2007**, *43*, 2080–2087.

(5) Rostoytsey, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596–2599.

(6) Binder, W. H.; Sachsenhofer, R. *Macromol. Rapid Commun.* **2007**, *28*, 15–54.
(7) Binder, W. H.; Sachsenhofer, R. *Macromol. Rapid Commun.* **2008**, *29*, 952–981.
(8) Shi, Q.; Chen, X.; Lu, T.; Jing, X. *Biomaterials* **2008**, *29*, 1118–1126.
(9) Ranjan, R.; Berittain, W. J. *Macromolecules* **2007**, *40*, 6217–6223.
(10) Guo, J.; Huang, Y.; Jing, X.; Chen, X. *Polymer* **2009**, *50*, 2847–2855.
(11) Agut, W.; Taton, D.; Lecommandoux, S. *Macromolecules* **2007**, *40*, 5653–5661.
(12) Ornelas, C.; Weck, M. *Chem. Commun.* **2009**, 5710–5712.
(13) Johnson, J. A.; Finn, M. G.; Koberstein, J. T.; Turro, N. J. *Macromol. Rapid Commun.* **2008**, *29*, 1052–1072.
(14) Fournier, D.; Hoogenboom, R.; Schubert, U. S. *Chem. Soc. Rev.* **2007**, *36*, 1369–1380.
(15) Cooper, B. M.; Chan-Seng, D.; Samanta, D.; Zhang, X.; Parelkar, S.; Emrick, T. *Chem. Commun.* **2009**, 815–817.
(16) Such, G. K.; Tjipto, E.; Postma, A.; Johnston, A. P. R.; Caruso, F. *Nano Lett.* **2007**, 1706–1710.
(17) Pierrat, P.; Vanderheiden, S.; Muller, T.; Bräse, S. *Chem. Commun* **2009**, 1748–1750.
(18) De Geest, B. G.; van Camp, W.; Du Prez, F. E.; De Smedt, S. C.; Demeester, J.; Hennink, W. E. *Chem. Commun.* **2008**, 190–192.
(19) Zhang, Z.; Zhou, Y.; Zhu, Z.; Ge, Z.; Liu, S. *Macromolecules* **2008**, *41*, 1444–1454.
(20) Sumerlin, B. S.; Vogt, A. P. *Macromolecules* **2010**, *43*, 1–13.
(21) Lutz, J.-F. *Angew. Chem., Int. Ed.* **2007**, *46*, 1018–1025.
(22) Iha, R. K.; Wooley, K. L.; Nyström, A. M.; Burke, D. J.; Kade, M. J.; Hawker, C. J. *Chem. Rev.* **2009**, *109*, 5620–5686.
(23) Horne, W. S.; Yadav, M. K.; Stout, C. D.; Ghadiri, M. R. *J. Am. Chem. Soc.* **2004**, *126*, 15366–15367.
(24) Horne, W. H.; Stout, C. D.; Ghadiri, M. R. *J. Am. Chem. Soc.* **2003**, *125*, 9372–9376.
(25) Qin, A.; Lam, J. W. Y.; Tang, B. *Chem. Soc. Rev.* **2010**, *39*, 2522–2544.
(26) Tsarevsky, N. V.; Sumerlin, B. S.; Matyjaszewski, K. *Macromolecules* **2005**, *38*, 3558–3561.
(27) Besset, C.; Binauld, S.; Ibert, M.; Fuertes, P.; Pascault, J.-P.; Fleury, E.; Bernard, J.; Drockenmuller, E. *Macromolecules* **2010**, *43*, 17–19.
(28) Yu, T.; Bai, J.; Guan, Z. *Angew. Chem., Int. Ed.* **2009**, *48*, 1097–1101.
(29) Hartwig, S.; Hecht, S. *Macromolecules* **2010**, *43*, 242–248.
(30) Binauld, S.; Damiron, D.; Hamaide, T.; Pascault, J.-P.; Fleury, E.; Drockenmuller, E. *Chem. Commun.* **2008**, 4138–4140.
(31) van Dijk, M.; Mustafa, K.; Dechesne, A. C.; van Nostrum, C. F.; Hennink, W. E.; Rijkers, D. T. S.; Liskamp, R. M. J. *Biomacromolecules* **2007**, *8*, 327–330.
(32) van Dijk, M.; Nollet, M. L.; Weijers, P.; Dechesne, A. C.; van Nostrum, C. F.; Hennink, W. E.; Rijkers, D. T. S.; Liskamp, R. M. J. *Biomacromolecules* **2008**, *9*, 2834–2843.
(33) Liu, X.; Thakur, A.; Wang, D. *Biomacromolecules* **2007**, *8*, 2653–2658.
(34) Wang, W.; Li, T.; Yu, T.; Zhu, F. *Macromolecules* **2008**, *41*, 9750–9754.
(35) van Steenis, D. J. V. C.; David, O. R. P.; Van Strijdonck, G. P. F.; Van Maarseveen, J. H.; Reek, J. N. H. *Chem. Commun.* **2005**, 4333–4335.
(36) Gao, Y.; Chen, L.; Zhang, Z.; Gu, W.; Li, Y. *Biomacromolecules* **2010**, *11*, 3102–3111.
(37) Qin, A.; Lam, J. W. Y.; Tang, L.; Jim, C. K. W.; Zhao, H.; Sun, J.; Tang, B. *Macromolecules* **2009**, *42*, 1421–1424.
(38) Nagao, Y.; Takasu, A. *Macromol. Rapid Commun.* **2009**, *30*, 199–203.
(39) Liu, X.-M.; Quan, L.-D.; Tian, J.; Laquer, F. C.; Ciborowski, P.; Wang, D. *Biomacromolecules* **2010**, *11*, 2621–2628.
(40) Carbini, B.; Benalil, A.; Vaultier, M. *J. Org. Chem.* **1993**, *58*, 3736–3741.
(41) Binder, W. H.; Sachsenhofer, R. *Macromol. Rapid Commun.* **2008**, *29*, 1097–1103.
(42) Knott, J.; Rossbach, V. *Angew. Makromol. Chem.* **1980**, *86*, 203–213.
(43) Hirayama, C.; Sakata, M.; Nakamura, M.; Ihara, H.; Kunitake, M.; Todokoro, M. *J. Chromatogr., B* **1999**, *721*, 187–195.
(44) Sakata, M.; Todokoro, C.; Hirayama, C. *Am. Biotechnol. Lab.* **2002**, *20*, 36 and 38.
(45) Hirayama, C.; Sakata, M. *J. Chromatogr., B* **2002**, *781*, 419–432.