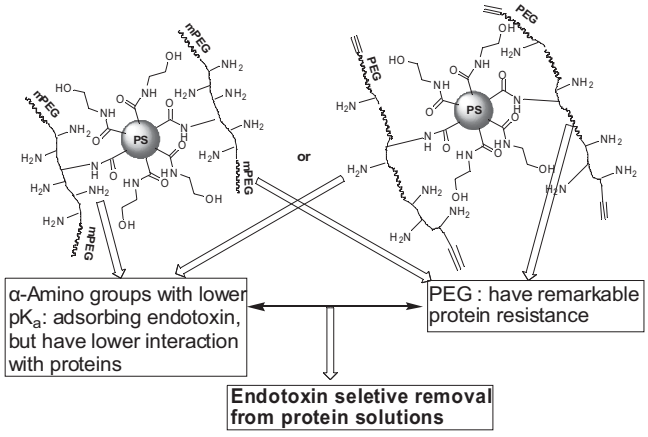
PEGylated Click Polypeptides Synthesized by Copper-Free Microwave-Assisted Thermal Click Polymerization for Selective Endotoxin Removal from Protein Solutions[a]

Jinshan Guo, Fanbo Meng,* Xiaoyuan Li, Mingzhe Wang, Yanjuan Wu, Xiabin Jing, Yubin Huang*

PEGylated click polypeptides (**PEG-CPs**) containing α-amino side groups as well as PEG segments are designed for selective endotoxin removal from protein solutions. The **PEG-CPs** are synthesized via copper-free thermal click copolymerization from aspartic (or glutamic) acid-based dialkyne and diazide monomers (containing free amino side groups) and alkyne-terminated mPEGs or dialkyne-terminated PEGs. Microwave-assisting technology is introduced into thermal click chemistry to improve the reaction efficiency. The monomers and polymers are fully characterized using NMR, XPS, and MALDI-TOF MS. After immobilizing the PEGylated click polypeptides onto polystyrene microspheres, the adsorbents exhibit good endotoxin removal selectivity from BSA solutions.



1. Introduction

Endotoxins (ETs) (lipopolysaccharides, LPS), a constituent of cell wall of gram-negative bacteria, largely exist in biological products, such as peptides and proteins. The removal of ET, even in nanogram quantities, from drugs and fluids before injections is critical because of the potent biological activities causing pyrogenic and shock reactions in mammals.[1,2] ETs are amphipathic molecules containing both anionic and hydrophobic regions. Consequently, the removal of ET often involves cationic ligands immobilized adsorbents.[2] The removal of ET from protein solutions, especially from acidic proteins such as bovine serum albumin (BSA), is extremely difficult because the common cationic ligands such as polymyxin,[3] histidine,[4] lysine,[5] diamine,[6] or chitosan,[7] show high adsorbing activities for both ETs and proteins. In view of this, Sakata et al., developed a series of biosynthesized poly(ε-lysine) (ε-PL) based adsorbents.[1,2] These adsorbents exhibited excellent selective ET removal properties from protein solutions,

Dr. J. Guo, X. Li, M. Wang, Y. Wu, Prof. X. Jing, Prof. Y. Huang
State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, No. 5625 Renmin Str., Changchun 130022, China
E-mail: ybhuang@ciac.jl.cn
Prof. F. Meng
The Cardiology Department of China-Japan Union Hospital of Jilin University, No. 126 Xiantai Str., Changchun 130033, China
E-mail: mengfb@jlu.edu.cn

[a] **Supporting Information** for this article is available from the Wiley Online Library or from the author.

DOI: 10.1002/mabi.201100394

owing to the low pK_a value (7.6) of ε-PL. The low pK_a value ensures the weak interaction between adsorbent and protein, even for acidic proteins. However, the large-scale production of ε-PL using biosynthesis techniques is inconvenient, the purification procedures are complicated, and the molecular weight of ε-PL is not easy to control. From a chemical standpoint, we have already developed a novel route for the chemosynthesis of the ε-PL-analog polymers using microwave-assisted copper (I)-catalyzed 1,3-dipolar azide/alkyne cycloaddition (CuAAC). The resulting triazole rings could perfectly imitate the functions of amide bonds even including spatial structures for the similar atomic spacing and dipole between the two.[8]

However, the Cu ions residuals after CuAAC click reaction severely limit its use in the biomedical areas concerning proteins, cells and in vivo applications.[8–15] Several kinds of copper-free click chemistry had been developed to avoid this concern, including ring strain or electron-withdrawing promoting [3 + 2] dipolar cycloaddition,[15–18] 1,3-dipolar cycloaddition of nitrile oxides and alkynes,[19] tetrazine/*trans*-cyclooctene cycloaddition,[20,21] Staudinger ligation,[22,23] Michael addition,[24] thiol-ene reaction,[25,26] and thiol-yne reaction.[26] However, most of the reactants used in these reactions are not easy to synthesize. Inorganic compounds or polymers supported copper catalysts[27–30] as well as nanoscale copper oxides[31,32] have been used for copper catalysts recycle or removal, but this approach decreases the Cu ion residuals presenting in products, which does not resolve the root problem. Recent studies re-examined the thermal 1,3-dipolar azide/alkyne cycloaddition (AAC, also named thermal click chemistry) without using copper catalysts, which was investigated much earlier than CuAAC.[33,34]

In this work, microwave-assisting technology was introduced into thermal click chemistry for the first time to improve the reaction efficiency and shorten the reaction time. A series of PEGylated click polypeptides containing α-amino acid groups were synthesized and immobilized onto functional polystyrene (PS) microspheres for the application of selective ET removal from protein solutions (Scheme 1 and 2). PEGylation was employed because PEG is a perfect anti-fouling agent with marked protein resistance.[35–37]

2. Experimental Section

2.1. General

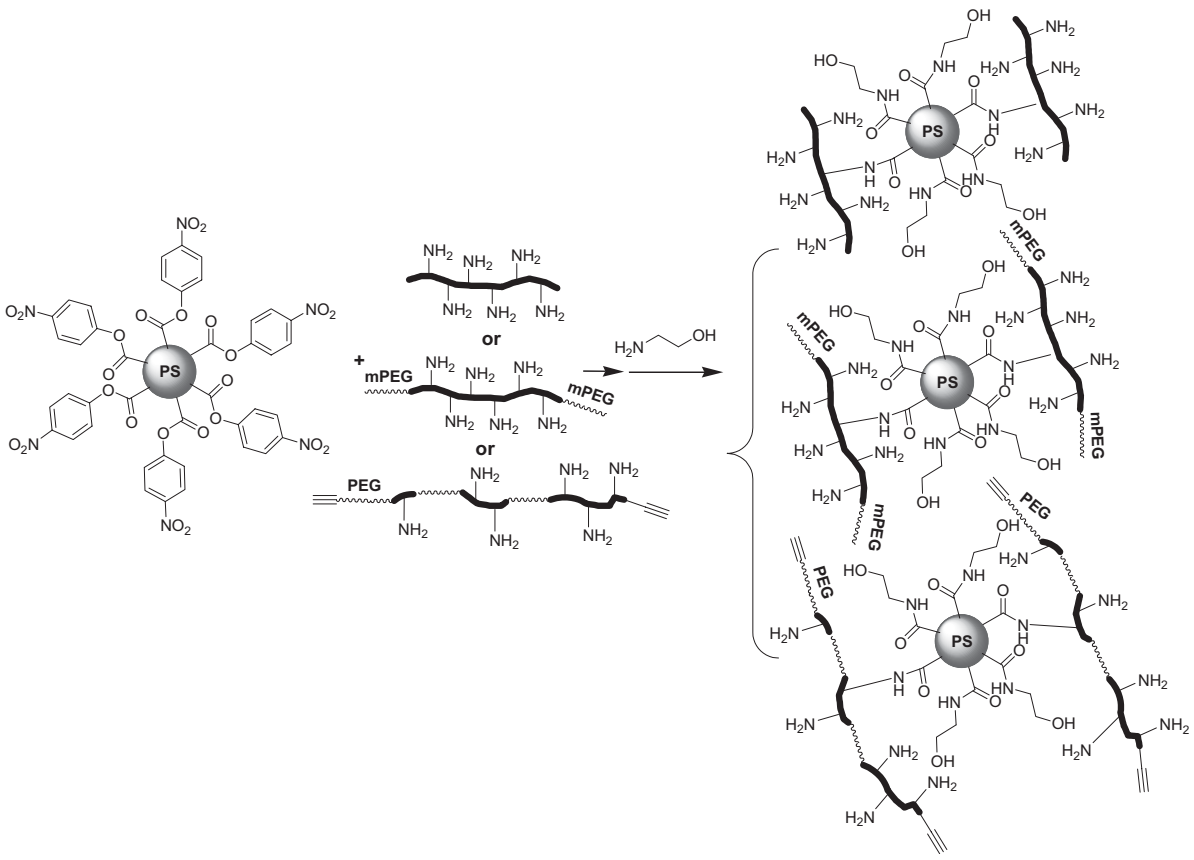
N-t-Butoxycarbonylaspartic acid (Boc-Asp), *N-t*-butoxycarbonyl-glutamic acid (Boc-Glu), BOP reagent (benzotrialzol-1-yl-oxytris-dimethylamino-phosphonium hexafluorosphosphate), and trifluoroacetic acid (CF_3COOH) were purchased from GL Biochem Ltd. (Shanghai, China). 3-Azidopropanamine was synthesized as described previously.[37,38] Poly(ethylene glycol) methyl ether (mPEG) with $\overline{M}_n$ of 1000 (**1a**), 2000 (**1b**), and 5000 (**1c**) as well as poly(ethylene glycol) (PEG) with average $\overline{M}_n$ of 950–1050 (**2a**) and 1900–2200 (**2b**), respectively were purchased from Sigma-Aldrich. Methacryoyl chloride (95% tech.) was purchased from Aladdin Reagent Inc (Shanghai, China) and used without further purification. ε-PL was provided by Zhejiang Silver-Elephant Bioengineering Co., Ltd. Standard 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^{-1}) and TAL (LAL) reagent water (ET concentration <0.005 EU · mL^{-1}) were from Zhanjiang Bokang Ocean Creature Company Ltd. BSA fraction V (BSA, 99%) was from Sigma-Aldrich. Propargylamine was purchased from Tianzunzezhong Chemical Limited Corporation (Nanjing, China). Copper (I) oxide (Cu_2O, 99.9%) and acetyl chloride were from Sigma-Aldrich and used without further purification. Copper (I) bromide (CuBr) 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. Bipyridine (BiPy) was purchased from Aladdin Reagent Inc. (Shanghai, China), and recrystallized with cyclohexane before use. Dichloromethane (DCM), triethylamine (TEA), and ethyl acetate were 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 Ltd., Zhengzhou, China). ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance DRX 300, 400 or 600 spectrometer in D_2O, $CDCl_3$ or DMSO-d_6. Quantitative ^{13}C NMR spectra were recorded on a Bruker Avance DRX 600 in D_2O and accumulated for more than 12 h (NS > 7 000). Matrix-assisted laser desorption/ionization time-of-flight mass spectra (MALDI-TOF MS) were recorded on an AutoflexIII Smart Beam Mass-spectrometer of Bruker Daltonics Inc without matrix except the liquid sample (diazido Glu, using 2,5-dehydroxybenzoic acid as matrices). X-ray photoelectron spectroscopy (XPS) was taken on a VG ESCALAB MK II electron spectrometer using Mg KR (1253.6 eV) as the X-ray excitation source at room temperature. Concentration of ET was assayed by a Limulus test involving turbidimetric time assay at 450 nm with Toxinometer BET-16 (Tianda Tianfa, Tianjin, China) at 37 °C. BSA concentration was determined by absorbence at 280 nm using a UV-2450 spectrometer (Shimadzu, Japan) with minimum wavelength resolution of 0.2 nm.

2.2. Synthesis of Dialkyne and Diazide Monomers with Free Amino Groups

2.2.1. Synthesis of Aspartic Dipropargyldiamide (Dipropargyl Asp) (**3**)

Dipropargyl Asp (**3**) was synthesized by the amide condensation of Boc-Asp with propargylamine, followed by the de-protection procedure of Boc group [Scheme 2(a)]. Firstly, Boc-Asp (2.33 g, 10 mmol) and dried TEA (8 mL, 56 mmol) were dissolved in 60 mL of dried DCM under nitrogen atmosphere with stirring. After addition of propargylamine (2.8 mL, 40 mmol) and BOP reagent (10.60 g,



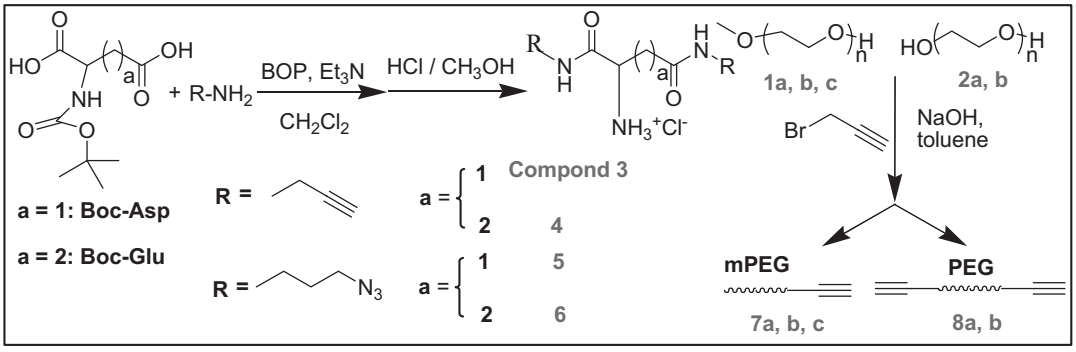
Scheme 1. The immobilization of polycations onto the crosslinked PS microspheres (the remained activated ester groups on microspheres were blocked by reacting with excess amount of ethanolamine).

24 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 ethyl acetate (EtAc). The EtAc solution was washed for three times with 1 M $KHSO_4$, once with H_2O, three times with 5% $NaHCO_3$, once again with H_2O, and three times with brine. During the wash procedure, the formed precipitate was removed by filtration. The organic layer was dried ($MgSO_4$), filtered, evaporated in vacuo, and finally dried under vacuum at 40 °C for 24 h to give dipropargyl Boc-Asp as a pale yellow solid (2.58 g, yield 83%), which was directly used in de-protection step. ^{1}H NMR (300 MHz; DMSO-d_6, δ): 1.38 [9H, br, (CH3)3CHOCO], 2.4 (2H, m, CHC**H**$_2$CO), 3.10 (2H, d, C≡C**H**), 3.83 (4H, br, C**H**$_2$C≡CH), 4.30 (1H, t, C**H**CO), 6.87–6.89 (1H, d, CHN**H**), 8.21–8.27 (2H, m, 2 CH_2CON**H**).

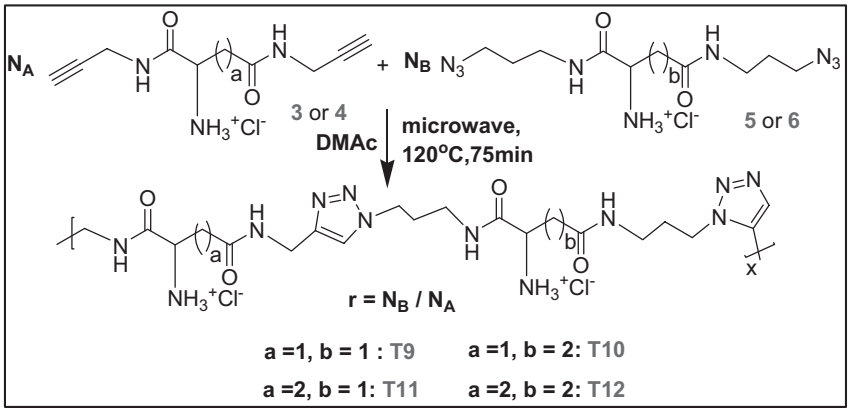
Secondly, the Boc groups of dipropargyl Boc-Asp were de-protected. Two different methods were used. The first method was de-protection with CF_3COOH. Typically, Boc-Asp (1.54 g, 5 mmol) was dissolved in 40 mL $CF_3COOH/CHCl_3$ (v/v = 1:1), and stirred for 2.5–3 h at room temperature. After the solvent was removed under vacuum, a little amount of hydrochloride acid was added to the system, and the residue was co-evaporated for three times with toluene and three times with chloroform. The viscous solid was precipitated in diethyl ether, filtered, and washed with diethyl ether. After dried under vacuum at 40 °C for 48 h, a pale yellow solid was obtained (1.10 g, yield 91%). Another de-protection way was using anhydrous HCl solution in methanol adapted from published method.[39] Acetylchloride (8.5 mL) was carefully added to methanol (73 mL) at 0 °C to give a 5 M solution of anhydrous HCl in MeOH. Then dipropargyl Boc-Asp (2.1 g, 6.83 mmol) was dissolved in the HCl solution in MeOH, and the mixture was stirred for 4 h at 0 °C. After the solvent was removed, the residue was co-evaporated for three times with toluene and three times with chloroform. The viscous solid was precipitated in diethyl ether, filtered, and washed with diethyl ether. After drying under vacuum at 40 °C for 48 h, a pale yellow solid was obtained (1.53 g, yield 92%). ^{1}H NMR (Figure S1 of Supporting Information) (300 MHz; DMSO-d_6, δ): 2.72 (2H, t, CHC**H**$_2$CO), 3.15, 3.18 (2H, 2t, C≡C**H**), 3.87–3.92 (4H, br, C**H**$_2$C≡CH), 4.07 (1H, t, C**H**CO), 8.28 (3H, s, $NH_3^+Cl^-$), 8.73, 8.94 (2H, 2t, 2 CH_2CON**H**). ^{13}C NMR (Figure S2 of Supporting Information) (101 MHz; DMSO-d_6, δ): 27.9, 28.3 (**C**H$_2$C≡CH), 35.4 (CH**C**H$_2$CO), 49.1 (**C**HCO), 73.3, 73.6 (C≡**C**H), 80.2, 80.7 (**C**≡CH), 167.5, 168.1 (NH**C**O). MALDI-TOF MS (Figure S3 of Supporting Information), $C_{10}H_{13}N_3O_2$ $[M + Na]^+$ Calcd. 230.09, observed 230.10; $[M + K]^+$ Calcd. 246.06, observed 246.10.

2.2.2. Synthesis of Glutamic Dipropargyldiamide (Dipropargyl Glu) (**4**)

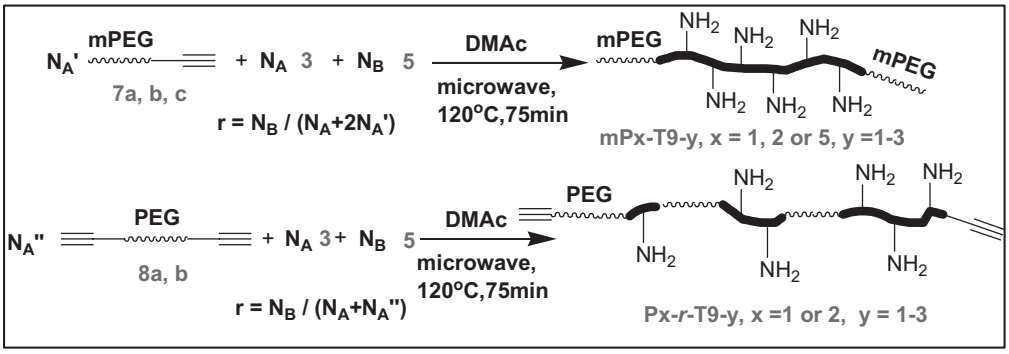
Compound **4** was synthesized by the amide condensation of Boc-Glu and propargylamine, followed by Boc de-protection, according



(a) Synthesis of monomers **3-6**, **7a, b, c** and **8 a,b**



(b) Microwave-assisted thermal click polymerization



(c) Click polypeptides with terminated mPEG and random-distributed PEG

Scheme 2. Microwave-assisted thermal click polymerization of diazide and dialkyne monomers **3–6**, accompany with or without alkyne-terminated mPEG **7a**, **7b**, **7c**, or dialkyne-terminated PEG **8a**, **8b**.

to the same procedure for the synthesis of compound **3** with a total yield of 78%. ^{1}H NMR (Figure S1 of Supporting Information) (300 MHz; DMSO-d_6, δ): 1.94–1.97 (2H, dt, CHC**H**$_2$CH$_2$CO), 2.20 (2H, t, CHCH$_2$C**H**$_2$CO), 3.00–3.07 (2H, m, C≡C**H**), 3.80–3.85 (4H, m, C**H**$_2$C≡CH), 3.94 (1H, t, C**H**CO), 8.37 (3H, s, NH_3^+Cl$^-$), 8.50, 9.10 (2H, 2t, 2 CON**H**). ^{13}C NMR (Figure S2 of Supporting Information) (101 MHz; DMSO-d_6, δ): 26.9 (**C**H$_2$CH$_2$CO), 27.9, 28.1 (**C**H$_2$C≡CH), 30.4 (**C**H$_2$CO), 51.6 (**C**HCO), 72.9, 73.7 (C≡**C**H), 80.3, 81.1 (**C**≡CH), 168.0, 170.6 (NH**C**O). MALDI-TOF MS (Figure S3 of Supporting Information), $C_{11}H_{15}N_3O_2$ $[M+Na]^+$ calcd. 244.11, observed 244.10; $[M+K]^+$ Calcd. 160.08, observed 260.10.

2.2.3. Synthesis of Aspartic Di(3-azidopropyl)diamide (Diazido Asp) (**5**)

Compound **5** was synthesized by the amide condensation of Boc-Asp and 3-azidopropanamine, followed by Boc de-protection, according to the same procedure for the synthesis of compound **4** with a total yield of 80%. Here, 3-azidopropanamine was threefolds excess. ^{1}H NMR (Figure S1 of Supporting Information) (300 MHz; DMSO-d_6, δ): 1.68 (4H, m, C**H**$_2$CH$_2$N$_3$), 2.71 (2H, m, CHC**H**$_2$CO), 3.12 (4H, t, C**H**$_2$N$_3$), 3.37 (4H, t, C**H**$_2$CH$_2$CH$_2$N$_3$), 4.05 (1H, t, C**H**CO), 8.32 (3H, s, NH_3^+Cl$^-$), 8.43, 8.66 (2H, 2t, 2 CON**H**). ^{13}C NMR (Figure S2 of

Supporting Information) (101 MHz; DMSO-d_6, δ): 28.3 (**C**$H_2CH_2N_3$), 35.8 (**C**H_2NH), 36.0 (CH**C**H_2COU), 48.4 (**C**H_2N_3), 50.0 (**C**HCO), 167.7, 168.5 (NH**C**O). MALDI-TOF MS (Figure S3 of Supporting Information), $C_{10}H_{19}N_9O_2$ $[]M+Na]^+$ Calcd. 320.16, observed 320.10; $[M+K]^+$ Calcd. 336.13, observed 336.10.

2.2.4. Synthesis of Glutamic Di(3-azidopropyl)diamide (Diazido Glu) (**6**)

Compound **6** was synthesized by the amide condensation of Boc-Glu and 3-azidopropanamine, followed by Boc de-protection, according to the same procedure for the synthesis of compound **4** with a total yield of 84%. The product was a viscous yellow liquid. Here, 3-azidopropanamine was also threefolds excess. ^{1}H NMR (Figure S1 of Supporting Information) (300 MHz; DMSO-d_6, δ): 1.58–1.92 (4H + 2H, m, C**H**$_2$CH$_2$N$_3$ and CHC**H**$_2$CH$_2$CO), 2.13 (2H, t, CHCH$_2$C**H**$_2$CO), 3.03–3.15 (4H, 2t, C**H**$_2$N$_3$), 3.35 (4H, t, C**H**$_2$CH$_2$CH$_2$N$_3$), 3.71 (1H, t, C**H**CO), 8.06 (1H, t, CH$_2$CON**H**), 8.24 (3H, s, N$H_3^+Cl^-$), 8.59 (1H, t, CHCON**H**). ^{13}C NMR (Figure S2 of Supporting Information) (101 MHz; DMSO-d_6, δ): 26.9 (**C**H_2CH$_2$COU), 28.3 (**C**H_2CH$_2$N$_3$), 30.6 (**C**H_2CO), 36.0 (**C**H_2NH), 48.3 (**C**H_2N$_3$), 52.2 (**C**HCO), 168.2, 171.0 (NH**C**O). MALDI-TOF MS (Figure S3 of Supporting Information), $C_{11}H_{22}N_9O_2$ $[M]^+$ Calcd. 312.19, observed 312.20.

2.2.5. Synthesis of Alkyne-Terminated mPEG (**7a**, **7b**, **7c**)

Alkyne-terminated mPEGs (**7a**, **7b**, **7c**) were synthesized adapting synthetic procedure described previously [Scheme 2(a)].[40] Generally, one equiv. of mPEG (**1a**, **1b**, **1c**) was reacted with the excess amount of propargyl bromide (20 equiv.) and NaOH powder (20 equiv.) in toluene for 15 h at 50 °C. The solvent was removed under vacuum and the residue was dissolved in water. The solution was extracted with DCM for twice. After dried with $MgSO_4$, the final product was obtained by precipitation in diethyl ether with a yield around 85%. NMR date for **7b** (Figure S4 of Supporting Information): ^{1}H NMR (300 MHz; $CDCl_3$, δ): 2.45 (t, C≡C**H**), 3.38–3.42 (s, OC**H**$_3$), 3.65–3.73 (br, C**H**$_2$C**H**$_2$O), 4.21 (d, C**H**$_2$C≡CH). ^{13}C NMR (75 MHz; CDCl3, δ): 58.6 (**C**H$_3$O), 59.2 (**C**H$_2$C≡CH), 70.8 (**C**H$_{2U}$**C**H$_2$O), 75.1 (C≡**C**H), 79.9 (**C**≡CH).

2.2.6. Synthesis of Dialkyne-terminated PEG (**8a**, **8b**)

Dialkyne-terminated PEGs (**8a**, **8b**) were synthesized as described in our previous work [Scheme 2(a)].[8] The average molecular weights of **6a** and **6b** were calculated from ^{1}H NMR as 1025 and 2152 g · mol^{-1}, respectively.

2.3. Microwave-Assisted Thermal Click Polymerizations

The molar ratios (r in Scheme 2) of diazide monomer **5** or **6** to dialkyne monomer **3** or **4** were designed to be 0.98 for all of the thermal click polypeptides to control the molecular weights of obtained polymers.

2.3.1. Synthesis of Thermal Click Polypeptide **9** (**T9**)

The synthesis of thermal click polypeptides was carried out in a microwave reactor at 120 °C without any catalyst [Scheme 2(b)]. Typically, a solution of dipropargyl Asp (compound **3**, 122 mg, 0.5 mmol) and diazido Asp (compound **5**, 163.5 mg, 0.49 mmol) in N_2-purged *N,N*-dimethylacetamide (DMAc, 1 mL) was placed in the microwave reactor and irradiated at 120 °C for 75 min under nitrogen atmosphere. After reaction, the solution was poured into diethyl ether (50 mL). The precipitant was centrifuged, resolved in methanol and precipitated in diethyl ether. The purification procedure was repeated twice. Then, the precipitant was washed with diethyl ether and dried under vacuum at room temperature for 48 h, **T9** as a yellow solid was obtained (257 mg, yield 90%). The molecular weight of **T9** was determined by quantitative ^{13}C NMR (Figure 1). ^{1}H NMR (300 MHz; D_2O, δ): 1.73, 2.06 (4H, d, CH$_2$C**H**$_2$CH$_2$NH), 2.85–2.96 (4H, br, CHC**H**$_2$CO), 3.12–3.32 [4H, br, triazole-(CH2)2 CH2NH], 3.76, 3.83 (br, 2xH, 1,5-triazole-C**H**$_2$NH), 4.24–4.42 [{[8–2x] + 2}H, br, COCH, 1,4-triazole-CH$_2$NH, triazole-CH$_2$ (CH$_2$)$_2$ NH], 7.56 (2xH, br, 1,5-triazole), 7.83 ([2–2x]H, s, 1,4-triazole) (Figure 1). ^{13}C NMR (quantitative) (151 MHz; D_2O, δ): 27.1, 28.4 (triazole-CH$_2$**C**H$_2$CH$_2$NH), 34.2, 35.1 [triazole-(CH2)2CH2NH], 36.1, 36.3 (CH**C**H$_2$CO), 47.3, 48.1 [triazole-CH$_2$NH and triazole-CH$_2$ (CH$_2$)$_2$NH], 49.7 (**C**HCO), 57.0 (2C, **C**≡CH of the terminal alkyne), 76.5

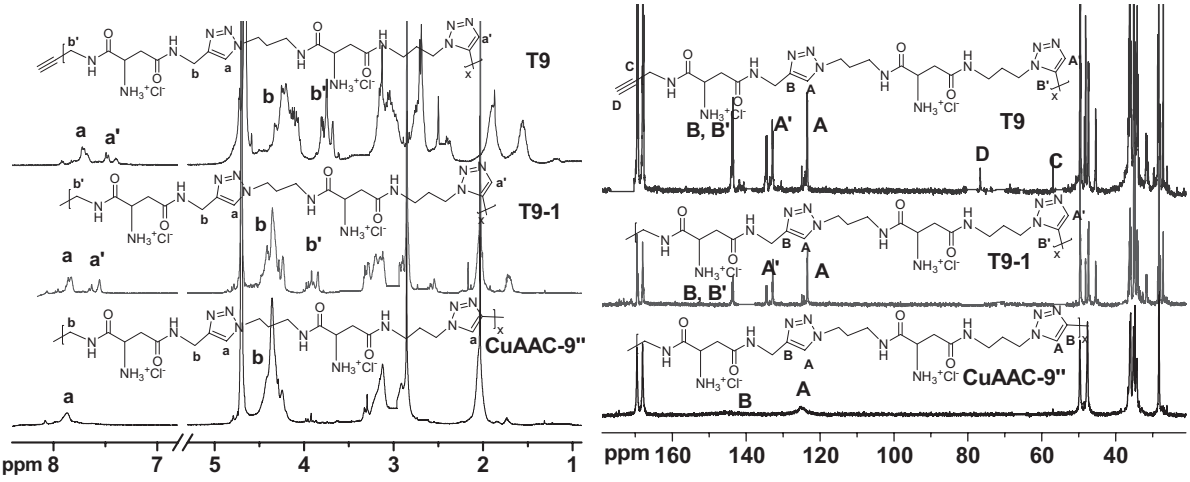


Figure 1. ^{1}H (left) and ^{13}C (right) NMR spectra of **CuAAC-9″**, **T9-1**, and **T9**.

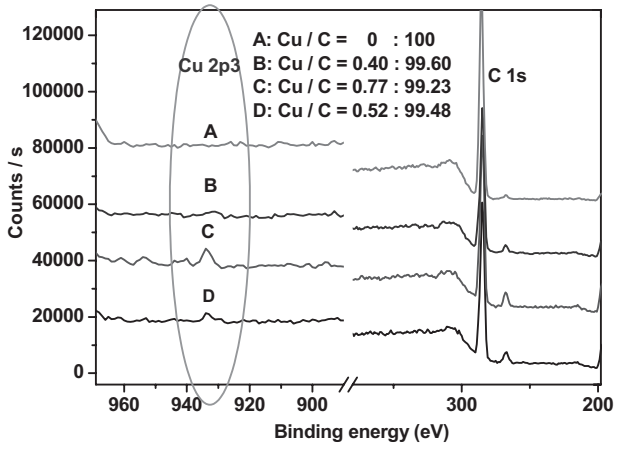


Figure 2. XPS of click polypeptides synthesized with or without copper catalyst. **A**: **T9** by microwave-assisted thermal click polymerization; **B**: **CuAAC-9″** (5 mg Cu_2O/0.5 mmol monomer); **C**: **CuAAC-9′** (10 mg Cu_2O/0.5 mmol monomer); **D**: **CuAAC-9** (7 mg CuBr/0.5 mmol monomer).

(C≡**C**H of the terminal alkyne), 123.5 (80.8 C, C=**C**H on 1,4-triazole), 132.9, 134.4, 134.6 (96.4 C, C=**C**H on 1,5-triazole), 143.5, 143.8 (**C**=CH on triazole), 168.0, 169.3, 169.5, and 171.2 (**C**ONH on the main chain). The copper constituent of **T9** was characterized by XPS (Figure 2).

2.3.2. Synthesis of Thermal Click Polypeptide **10** (**T10**)

Thermal click polypeptide **10** (**T10**) was obtained using dipropargyl Glu (compound **4**, 129 mg, 0.5 mmol) and diazido Asp (compound **5**, 163.5 mg, 0.49 mmol), according to the same procedure for the synthesis of **T9** with a yield of 91% (266 mg). ^{1}H NMR (300 MHz; D_2O, δ): 1.55–1.59, 1.87–2.00 (6H, d, $CH_2C\mathbf{H}_2CH_2NH$ and $C\mathbf{H}_2CH_2CO$), 2.23, 2.38 (2H, d, $CH_2C\mathbf{H}_2CO$), 2.67 (2H, m, $CHC\mathbf{H}_2CO$), 3.06–3.17 [4H, br, triazole-(CH2)2 CH2NH], 3.71, 3.83 (br, 2*x*H, 1,5-triazole-$C\mathbf{H}_2NH$), 4.06–4.28 [{[8−2*x*] + 2}H, br, COCH, 1,4-triazole-CH2NH, triazole-$CH_2(CH_2)_2NH$], 7.49 (2*x*H, br, 1,5-triazole), 7.75, 7.91 ([2−2*x*]H, s, 1,4-triazole). ^{13}C NMR (quantitative) (151 MHz; D_2O, δ): 26.8 (**C**H_2CH_2CO), 28.5 (triazole-CH_2**C**H_2CH_2NH), 31.8 (CH_2**C**H_2CO), 34.1, 35.7 [triazole-$(CH_2)_2$**C**H_2NH], 36.4 (CH**C**H_2CO), 46.9, 47.5, 48.5 [triazole-CH_2NH and triazole-$CH_2(CH_2)_2NH$], 49.4, 49.5 (**C**HCH_2CO), 56.6 [$CH(CH_2)_2CO$], 56.9 (2C, **C**≡CH of the terminal alkyne), 76.6 (C≡**C**H of the terminal alkyne), 123.5 (85.0 C, C=**C**H in 1,4-triazole), 132.8, 134.5 (94.0 C, C=**C**H in 1,5-triazole), 144.4 (**C**=CH in triazole), 167.9, 169.3, 171.3, and 174.6 (**C**ONH on the main chain).

2.3.3. Synthesis of Thermal Click Polypeptide **11** (**T11**)

Thermal click polypeptide **11** (**T11**) was obtained using dipropargyl Asp (compound **3**, 122 mg, 0.5 mmol) and diazido Glu (compound **6**, 170.4 mg, 0.49 mmol), according to the same procedure for the synthesis of **T9** with a yield of 92% (269 mg). ^{1}H NMR (300 MHz; D_2O, δ): 1.72–1.92 (6H, br, $CH_2C\mathbf{H}_2CH_2NH$ and $C\mathbf{H}_2CH_2CO$), 2.07, 2.21, 2.67–2.89 (4H, m, $CH_2C\mathbf{H}_2CO$ and $CHC\mathbf{H}_2CO$), 2.92–3.09 [4H, br, triazole-$(CH2)_2CH_2NH$], 3.67–3.79 (br, 2*x*H, 1,5-triazole-$C\mathbf{H}_2NH$), 4.07–4.32 [{[8−2*x*] + 2}H, br, COCH, 1,4-triazole-CH_2NH, triazole-$CH_2(CH2)_2NH$], 7.39, 7.49 (2*x*H, d, 1,5-triazole), 7.74 ([2−2*x*]H, s, 1,4-triazole). ^{13}C NMR (quantitative) (151 MHz; D_2O, δ): 27.1 (**C**H_2CH_2CO), 28.93 (triazole-CH_2**C**H_2CH_2NH), 30.1 (CH_2**C**H_2CO), 34.0, 35.1 [triazole-$(CH_2)_2CH_2NH$], 36.5 (CH**C**H_2CO), 47.2, 47.4, 48.1 [triazole-CH_2NH and triazole-$CH_2(CH_2)_2NH$], 49.7 (**C**HCH_2CO), 52.1 [$CH(CH_2)_2CO$], 56.9 (2C, **C**≡CH of the terminal alkyne), 76.1 (C≡**C**H of the terminal alkyne), 123.6, 124.9 (97.2 C, C=**C**H on 1,4-triazole), 132.8, 134.3, 134.8 (91.6 C, C=**C**H on 1,5-triazole), 144.0 (**C**=CH on triazole), 167.8, 168.4, 169.5 (**C**ONH on the main chain).

2.3.4. Synthesis of Thermal Click Polypeptide **12** (**T12**)

Thermal click polypeptide **12** (**T12**) was obtained using dipropargyl Glu (compound **4**, 129 mg, 0.5 mmol) and diazido Glu (compound **6**, 170.4 mg, 0.49 mmol), according to the same procedure for the synthesis of **T9** with a yield of 91% (272 mg). ^{1}H NMR (300 MHz; D_2O, δ): 1.56–1.71, 1.92 (8H, m, $CH_2C\mathbf{H}_2CH_2NH$ and $C\mathbf{H}_2CH_2CO$), 2.22 (4H, br, $CH_2C\mathbf{H}_2CO$), 3.03 [4H, br, triazole-$(CH_2)_2CH_2NH$], 3.72–3.80 (br, 2*x*H, 1,5-triazole-$C\mathbf{H}_2NH$), 4.22, 4.38 [{[8−2*x*] + 2}H, br, COCH, 1,4-triazole-CH_2NH, triazole-$CH_2(CH_2)_2NH$], 7.47 (2*x*H, d, 1,5-triazole), 7.76 ([2−2*x*]H, s, 1,4-triazole). ^{13}C NMR (quantitative) (151 MHz; D_2O, δ): 27.9 (**C**H_2CH_2CO), 28.9 (triazole-CH_2**C**H_2CH_2NH), 31.2 (CH_2**C**H_2CO), 36.7 [triazole-$(CH_2)_2CH_2NH$], 47.3, 47.5 [triazole-CH_2NH and triazole-$CH_2(CH_2)_2NH$], 53.4, 54.0 (CO**C**H), 57.0 (2C, **C**≡CH of the terminal alkyne), 76.0 (C≡**C**H of the terminal alkyne), 123.4, 125.0 (67.6 C, C=**C**H on 1,4-triazole), 130.6, 133.7, 134.7 (70.4 C, C=**C**H on 1,5-triazole), 144.0 (**C**=CH on triazole), 168.8, 173.2, 174.5 (**C**ONH on the main chain).

2.4. Cytotoxicity of Thermal Click Polypeptides

The relative cytotoxicity of **T9**, **T10**, **T11**, and **T12** was assessed with a methyl tetrazolium (MTT) assay against L929 Mouse fibroblast. Biosynthesized ε-PL and chemically synthesized poly(L-lysine) (PLL) were used as the positive and negative control, respectively. The weighed dry samples (ε-PL, **T9**, **T10**, **T11**, **T12**, and PLL) were sterilized by ultraviolet (UV) and then dissolved in Dulbecco's modified Eagle medium (DMEM, GIBCO). Subsequently, 100 μL of L929 cells in DMEM 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 addition of the sample-containing culture medium to make the final sample concentrations of 0.5, 0.25, 0.125, mg · mL^{-1}, respectively. After another 24 and 48 h of incubation, the cells were observed using an inverted microscope (Nikon-2000U). A 20 μL of MTT stock solution (5 mg · mL^{-1} in PBS) was added to each well of the plate for an additional 4 h of incubation. The purple formazan produced by active mitochondria was dissolved using 200 μL of dimethyl sulfoxide (DMSO). The optical density (OD) at 492 nm in each well was measured on a micro-plate reader. Each sample took at least four wells, and the average results were shown in Figure 3 and 4.

2.5. PEGylated Click Polypeptides (PEG-CPs)

2.5.1. Click Polypeptides with Terminated mPEG (**mPEG-CP**s)

Click polypeptides with terminated mPEG (**mP*x*-T9-*y*, *x*** = 1, 2, or 5, here 1, 2, and 5 represent for the molecular weights 1000, 2000, and 5000 of mPEGs, respectively; ***y*** = 1–3, here 1, 2, and 3 represent for

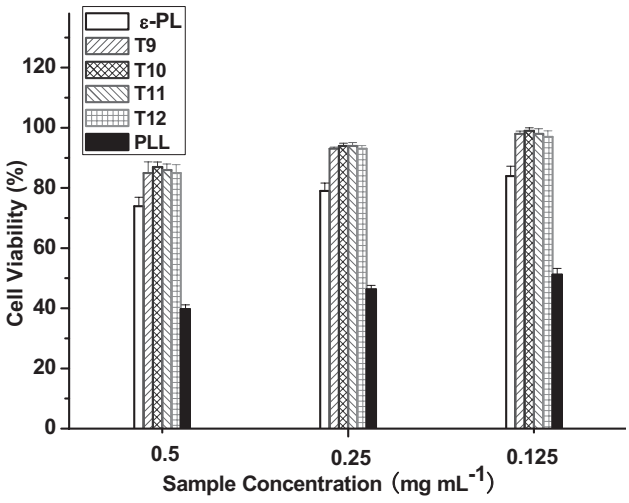


Figure 3. Cytotoxicity of ε-PL, **T9**, **T10**, **T11**, **T12**, and PLL at different polymer concentrations against L929 fibroblasts evaluated by MTT assay after 48 h.

the molar ratios 0.98, 0.94, and 0.90 of the reacting monomers, respectively) were synthesized by adding alkyne-terminated mPEGs (**7a**, **7b**, **7c**) to the polymerization system of dipropargyl Asp (**3**) and diazido Asp (**5**) [Scheme 2(c)]. The synthesis of **mP1-T9-1** ($r = 0.98$) is shown below as an example. Alkyne-terminated mPEG1000 (**7a**) (5.3 mg, 0.005 mmol), compound **3** (119.5 mg, 0.49 mmol) and compound **5** (163.5 mg, 0.49 mmol) were dissolved in N_2-purged DMAc (1 mL) and settled in the microwave reactor. The reaction mixture was irradiated at 120 °C for 75 min under a nitrogen atmosphere. After purification by precipitating and washing as described in the synthesis of **T9**, a yellow product was obtained after drying under vacuum with a yield around 89% (257 mg).

Because the structures of this series of polymers are similar, only the NMR data of **mP5-T9-1** was given below as an example (Figure 5).

NMR data of **mP5-T9-1**: ^{1}H NMR (600 MHz; D_2O, δ): 1.73, 2.05 (4H, d, $CH_2C\mathbf{H}_2CH_2NH$), 2.84–2.92 (4H, br, $CHC\mathbf{H}_2CO$), 3.13–3.33 [4H, m, triazole-$(CH_2)_2C\mathbf{H}_2NH$], 3.64 (6.0H, s, $C\mathbf{H}_2C\mathbf{H}_2O$ in mPEG), 3.84–3.96 (m, 2*x*H, 1,5-triazole-$C\mathbf{H}_2NH$), 4.22–4.48 [{[8–2*x*] + 2}H, br, COCH, 1,4-triazole-CH_2NH, triazole-$CH_2(CH_2)_2NH$], 7.55, 7.63 (2*x*H, d, 1,5-triazole), 7.87 ([2–2*x*]H, s, 1,4-triazole). ^{13}C NMR (151 MHz; D_2O, δ): 28.6 (triazole-$CH_2\underline{C}H_2CH_2NH$), 348, 35.2 ($CH\underline{C}H_2CO$), 36.0, 36.6 [triazole-$(CH_2)_2CH_2NH$], 47.3, 47.4, 48.2, 48.5 [triazole-CH_2NH and triazole-$CH_2(CH_2)_2NH$], 49.6, 49.7 ($CO\mathbf{C}H$), 69.1 ($\underline{C}H_2\underline{C}H_2O$ in mPEG), 72.2 ($\underline{C}H_3$– in mPEG), 123.5 (C=$\underline{C}$H on 1,4-triazole), 132.8 (C=$\underline{C}$H on 1,5-triazole), 143.5, 143.8 ($\underline{C}$=CH on triazole), 167.9, 169.6 ($\underline{C}$ONH on the main chain).

2.5.2. Click Polypeptides with Randomly Distributed PEG (**PEG-*r*-CPs**)

Click polypeptides with randomly distributed PEG (**P*x*-*r*-T9-*y*, *x*** = 1 or 2, here 1 and 2 represent for the molecular weights 1000 and 2000 of PEGs, respectively; ***y*** = 1–3, here 1, 2, and 3 represent for the

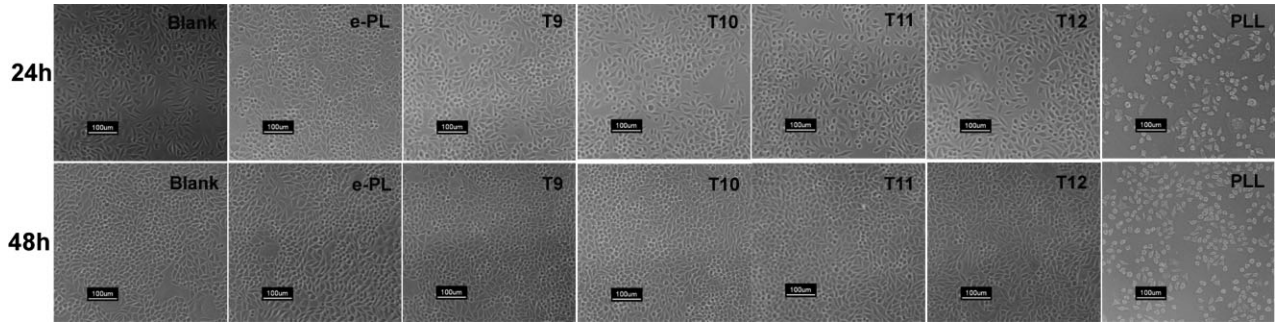


Figure 4. Optical images of the incubated L929 fibroblast cells. Blank: without polymer (blank); **e-PL**: in 0.5 mg · mL^{-1} of ε-PL; **T9**: in 0.5 mg · mL^{-1} of **T9**; **T10**: in 0.5 mg · mL^{-1} of **T10**; **T11**: in 0.5 mg · mL^{-1} of **T11**; **T12**: in 0.5 mg · mL^{-1} of **T12**; and **PLL**: in 0.125 mg · mL^{-1} of PLL for 24 and 48 h.

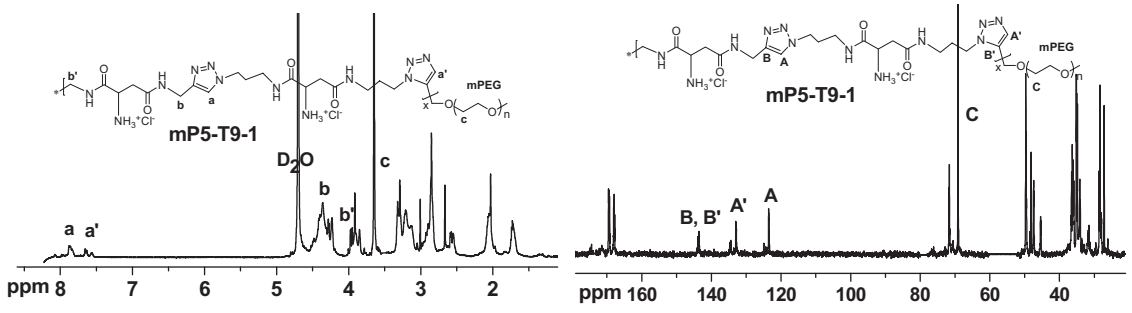


Figure 5. ^{1}H (left) and ^{13}C (right) NMR spectra of click polypeptide with terminated mPEG.

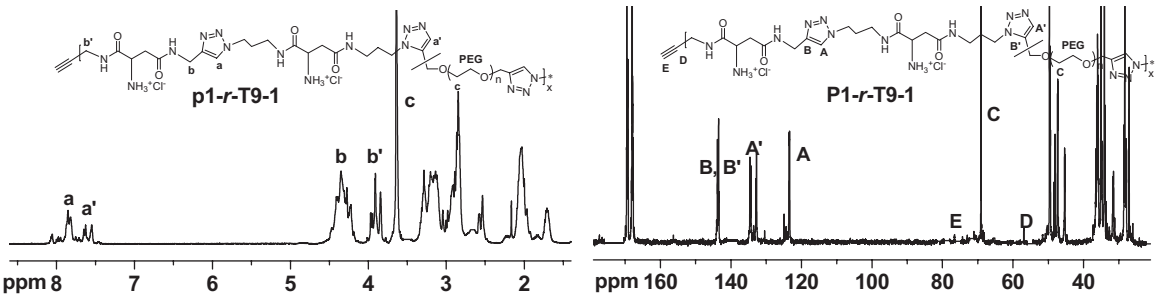


Figure 6. ^{1}H (left) and ^{13}C (right) NMR spectra of click polypeptide with random-distributed PEG.

molar ratios 0.98, 0.94 and 0.90 of the reacting monomers, respectively) were synthesized by adding dialkyne-terminated PEGs (**8a**, **8b**) to the polymerization system of dipropargyl Asp (**3**) and diazido Asp (**5**) [Scheme 2(c)]. The synthesis of **P1-*r*-T9-1** ($r = 0.98$) is shown below as an example. Dialkyne-terminated PEG1000 (10.3 mg, 0.01 mmol), compound **3** (119.5 mg, 0.49 mmol) and compound **5** (163.5 mg, 0.49 mmol) were dissolved in N_2-purged DMAc (1 mL) and then settled in the microwave reactor. The reaction mixture was irradiated at 120 °C for 75 min under a nitrogen atmosphere. After purification, a yellow product was obtained with a yield around 90% (264 mg).

Because the structures of this series of polymers are similar, only the NMR data of **P1-*r*-T9-1** was given below as an example (Figure 6).

NMR data of **P1-*r*-T9-1**: ^{1}H NMR (400 MHz; D_2O, δ): 1.73, 2.05 (4H, d, $CH_2CH_2CH_2NH$), 2.89–2.92 (4H, br, $CHCH_2CO$), 3.12–3.32 [4H, m, triazole-$(CH_2)_2CH_2NH$], 3.64 (5.4H, s, CH_2CH_2O in PEG), 3.84–3.97 (m, 2*x*H, 1,5-triazole-CH_2NH), 4.22–4.41 [{[8–2*x*] + 2}H, br, COCH, 1,4-triazole-CH_2NH, triazole-$CH_2(CH_2)_2NH$], 7.55, 7.65 (2*x*H, d, 1,5-triazole), 7.85 ([2–2*x*]H, s, 1,4-triazole). ^{13}C NMR (quantitative) (151 MHz; D_2O, δ): 27.1, 28.3 (triazole-$CH_2CH_2CH_2NH$), 34.1, 35.1 [triazole-$(CH_2)_2CH_2NH$], 36.0 ($CHCH_2CO$), 45.3, 47.3 [triazole-$CH_2(CH_2)_2NH$], 49.7 ($CHCO$), 57.2 ($C{\equiv}CH$ of the alkyne terminal), 65.5 (triazole-CH_2O), 69.1 (CH_2CH_2O of PEG), 76.0 ($C{\equiv}CH$ of the alkyne terminal), 123.5 (C=CH on 1,4-triazole), 132.9, 134.6 (C=CH on 1,5-triazole), 143.8 (C=CH on triazole), 168.0, 169.5 (CONH on the main chain).

The chain numbers of PEG1000 or PEG2000 in each polymer, which calculated by the combination of ^{1}H NMR and quantitative ^{13}C NMR, were shown below: **P1-*r*-T9-1**: 5.07, **P1-*r*-T9-2**: 4.13, **P1-*r*-T9-3**: 3.80, **P2-*r*-T9-1**: 5.24, **P2-*r*-T9-2**: 3.94, **P2-*r*-T9-3**: 3.96.

2.5.3. Synthesis of Thermal Click Polypeptide **9** with $r = 1$ (***T9-1***)

T9-1 was synthesized using equal amount of dipropargyl Asp (compound **3**) and diazido Asp (compound **5**) (for 1 mL of DMAc, 0.5 mmol **3**, and 0.5 mmol **5** were used), according to the same procedure for the synthesis of **T9** with a yield of 90% (260 mg). ^{1}H NMR (600 MHz; D_2O, δ): 1.69 (4H, d, $CH_2CH_2CH_2NH$), 2.88–2.97 (4H, br, $CHCH_2CO$), 3.02–3.35 [4H, br, triazole-$(CH_2)_2CH_2NH$], 3.66–4.09 (br, 2*x*H, 1,5-triazole-CH_2NH), 4.35–4.54 [{[8–2*x*] + 2}H, br, COCH, 1,4-triazole-CH_2NH, triazole-$CH_2(CH_2)_2NH$], 7.40, 7.71 (2*x*H, br, 1,5-triazole), 7.86 ([2–2*x*]H, s, 1,4-triazole). ^{13}C NMR (151 MHz; D_2O, δ): 27.1, 28.3 (triazole-$CH_2CH_2CH_2NH$), 34.7, 35.1 [triazole-$(CH_2)_2CH_2NH$], 36.6 ($CHCH_2CO$), 47.3, 47.4, 48.2 [triazole-CH_2NH and triazole-$CH_2(CH_2)_2NH$], 49.7 ($CHCO$), 123.5 (C=CH on 1,4-triazole), 132.9, 134.5 (C=CH on 1,5-triazole), 143.9 (C=CH on triazole), 168.2, 169.5, and 173.4 (CONH on the main chain).

2.5.4. Synthesis of CuAAC Click Polypeptide **9** (**CuAAC-9**)

The synthesis of CuAAC click polypeptide was carried out in a microwave reactor using CuBr or Cu_2O as catalyst. Generally, a solution of dipropargyl Asp (compound **3**, 122 mg, 0.5 mmol), diazido Asp (compound **5**, 167 mg, 0.5 mmol), CuBr (7 mg, 0.05 equiv.) and BiPy (31.2 mg, 0.2 equiv.) in N_2-purged DMAc (1 mL) was placed in the microwave reactor and was irradiated at 100 °C for 30 min under nitrogen atmosphere. When the catalyst was changed to Cu_2O (50 or 10 mg for the same amount of monomers) without using BiPy, the product was named as **CuAAC-9′** and **CuAAC-9″**, respectively. ^{1}H NMR of **CuAAC-9″** (Figure 1) (600 MHz; D_2O, δ): 2.02 (4H, br, 2 CH_2CH_2NH), 2.71–2.88 (4H, m, $CHCH_2CO$), 3.04–3.27 (4H, br, triazole-$CH_2CH_2CH_2NH$), 3.97, 4.05 (2H, d, COCH), 4.24–4.43 [8H, br, triazole-$CH_2(CH2)_2NH$ and triazole-CH_2NH], 7.84 (2H, br, the H in the two triazole). ^{13}C NMR (151 MHz; D_2O, δ): 28.3 (triazole-$CH_2CH_2CH_2NH$), 33.8, 35.2 [triazole-$(CH_2)_2CH_2NH$], 37.7 ($CHCH_2CO$), 47.6 [triazole-CH_2NH and triazole-$CH_2(CH_2)_2NH$], 49.7 ($CHCO$), 125.2 (C=CH on triazole), 147.4 (C=CH on triazole), 168.0, 169.6, 173.5 (CONH on the main chain). The copper contents of these polymers were determined by XPS (Figure 2).

2.6. Immobilization of Polycations Onto Crosslinked Polystyrene Microspheres

In order to assess the ET selective removal properties of PEGylated click polypeptides, crosslinked PS microspheres with activated ester groups (PS-AE) was prepared by dispersion polymerization of 4-nitrophenyl methacrylate (NPMA) (synthesis according to literature,[41] Scheme S1 of Supporting Information), styrene (S), and divinyl benzene (DVB) (see Supporting Information and Scheme S2 of Supporting Information). Different kinds of PEGylated click polypeptides, **T9**, ε-PL (as positive control) and chemically synthesized PLL (as negative control) were then immobilized to PS-AE microspheres through the reaction between amino groups and activated ester groups. After that, the remaining activated ester groups were blockaded by reacting with excess ethanolamine (Scheme 1). Typically, 25 mg of the PS-AE microspheres was

suspended in 1 mL ethanol/water (80:20 vol%) by ultrasonication, after that, 50 μL TEA was added. 5 mg of ε-PL (containing 39.1 μmol amino groups) was then dissolved in. The suspension was oscillated vigorously at 50 °C for 24 h under a nitrogen atmosphere. Then 1 mL of ethanolamine was added, and the reaction mixture was kept under 50 °C for another 4 h. The resulting ε-PL-immobilized crosslinked PS-AE microspheres was collected and washed several times with water and freeze-dried. **T9**, PLL or PEGylated click polypeptides immobilized crosslinked PS-AE microspheres were prepared by the same method. The amino-group contents of polycation immobilized PS-AE microspheres were determined by ninhydrin technique according to literature,[42] and the results are shown in Table 2.

2.7. Selective ET Removal Properties of PEGylated Click Polypeptides

The ET adsorption and protein recovery properties of polycation immobilized microspheres (for 1 mL ET or protein solution, 10 mg microspheres was used) were investigated independently. The ET adsorption process was described below: typically, the adsorbent was firstly treated with 0.2 M NaOH solution, normal saline, and ethanol to remove the original pyrogen and other impurities. The adsorbent was then dispersed ultrasonically in 1 mL ET solution in TAL (LAL) reagent water with a concentration of 1000 EU · mL^{-1}. The mixture was oscillated at 25 °C for 15 min. The adsorbent was centrifuged and the ET concentration of the supernatant was measured by a Limulus test. The protein recoveries of different polycation immobilized PS-AE microspheres were determined as below: after pretreatment as described in the ET adsorption process, the adsorbent was mixed with 1 mL 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 ET selective removal properties from BSA solution of different polycation immobilized PS-AE microspheres were determined as below: BSA solution that containing about 11 500 EU · mL^{-1} ET was prepared, and then the pretreated adsorbent was mixed with 1 mL the above mentioned solution. After oscillating at 25 °C for 15 min, the adsorbent was centrifuged. The ET and BSA concentration of the supernatant were measured. All the ET adsorption and protein recovery experiments were repeated for three times, and the results were averaged.

3. Results and Discussion

In our previous work,[8] a series of ε-PL (baring α-amino side groups)-analogous polymers were chemically synthesized using microwave-assisted click polymerization, and the polymers were named as click polypeptides. The primary advantage of these kind of click polypeptides (also have α-amino side groups) is their low pK_a values (around 7.5) as that of ε-PL (pK_a = 7.6), which is much lower than that of PLL (baring ε-amino side groups, pK_a = 9–10). The low pK_a minimizes the interaction between polymers with proteins, especially with acidic protein like BSA. This is a critical feature for the selective removal of ET from protein solutions. In this work, PEG segments which have protein resistance ability were also introduced to the click polypeptides to form PEGylated click polypeptides, expected to further improve the selectivity of ET adsorption from protein solutions. We also changed the polymer synthesis strategy by de-protecting the protected amino groups before polymerization. In order to get rid of the side effect of Cu ions residuals in the process of protein recovery, we used microwave-assisted thermal click polymerization instead of CuAAC for the polymerization. All these designs aimed at selective ET removal from protein solutions, especially acidic protein (such as BSA) solutions.

3.1. Monomer Synthesis

In our previous research,[8] dialkyne and diazide monomers were synthesized from benzyloxycarbonyl (Z) protected aspartic (or glutamic) acid. The Z group was de-protected after click polymerization. However, different strategy was applied in the present work. In this case, Boc-Asp or Boc-Glu was first reacted with 3-azidopropanamine or propargylamine. Then the de-protection of Boc groups was carried out right after the monomers synthesis to obtain the dialkyne and diazide monomers with free amino groups. These unprotected dialkyne and diazide monomers were then applied for the later click polymerizations. The change of the de-protection sequence not only made it easier to control the content of the amino groups in the later copolymers, but also lowered the risk of polymer degradation. Dialkyne and diazide monomers with amino side groups in the state of hydrochloride salt were obtained by treating the Boc-protected monomers with anhydrous HCl solution in methanol, which was formed by the reaction of acetylchloride and methanol according to literature.[39] Alkyne-terminated mPEG **7a**, **7b**, and **7c** as well as dialkyne-terminated PEG **8a**, **8b** with different molecular weights (molecular weight of **1a**, **1b**, and **1c** [mPEG] are 1000, 2000, and 5000 Da respectively, molecular weight of **2a** and **2b** [PEG] are 1000, 2000 Da, respectively) were synthesized by the reaction of mPEG or PEG with large excess of propargyl bromide (40-folds) using NaOH as catalyst [Scheme 2(a)]. All the diazide and dialkyne monomers **3–6** as well as alkyne-terminated mPEG **7a**, **7b**, **7c**, and dialkyne-terminated PEG **8a**, **8b** were characterized by ^{1}H and ^{13}C NMR (Figure S1, S2, and S4 in supporting information), indicating high purity. Monomers **3–6** were also confirmed by mass spectroscopy (Figure S3 in Supporting Information).

3.2. Thermal Click Polypeptides

The thermal click polypeptides **T9**-**T12** were synthesized through thermal click polymerizations between dialkyne **3**

or **4** and diazide **5** or **6** monomers. In order to control the molecular weights of polymers and make the characterization convenient, unequal equivalence of purified dialkyne **3** or **4** and diazide **5** or **6** monomers were mixed together in DMAc under nitrogen atmosphere without copper catalyst, and polymerization were processed in microwave reactor for 75 min. The monomers with free amino side groups can only dissolve in DMSO and DMAc. Since DMSO and diethyl ether are not mutually soluble, DMAc was chosen as solvent for the convenience of post-treatment using diethyl ether as precipitating solvent. According to the previous research[33,34,43] and our experiment results, the reaction time and temperature for microwave-assisted thermal click polymerization were optimized to be 75 min and 120 °C, respectively. After polymerizing for 75 min under 120 °C with microwave-assisting, the polymerization degrees kept consistent with design (Table 1). The obtained thermal click polypeptides **T9**-**T12** with two terminated alkyne groups were characterized by ^{1}H and quantitative ^{13}C NMR. Since the signal of terminated alkyne groups in ^{1}H NMR was very weak, and the SEC-MALLS analysis as well as MALDI-TOF MS spectra results gave no useful information about molecular weights for the free amino side groups in polymers, the polymerization degrees of **T9**-**T12** were determined by quantitative ^{13}C NMR (Table 1 and Figure 1).

As an example, ^{1}H and ^{13}C NMR spectra of thermal click polypeptide **T9**, **T9-1** (obtained using equal equivalence of monomers) and **CuAAC-9**″ (obtained using equal equivalence of monomers, with Cu_2O as catalyst) are presented in Figure 1. The polyaddition reactions between dialkyne and diazide monomers were confirmed by the appearance of the characteristic signals of triazole rings at $\delta = 7.56$ (1,5-triazole) and 7.83 (1,4-triazole) in ^{1}H NMR (Figure 1, left, **T9**, a, a′), as well as at $\delta = 123.5$ (C=**C**H on 1,4-triazole), 132.9, 134.4, 134.6 (C=**C**H on 1,5-triazole) and 143.5, 143.8 (**C**=CH on triazole) in the ^{13}C NMR spectra (Figure 1, right, **T9**, A, A′ and B, B′). The coexistence of 1,4-triazole and 1,5-triazole is the distinct characteristic of thermal click chemistry that is different from that of CuAAC. The signals of terminated alkyne groups of **T9** could be found in the ^{13}C NMR spectra (Figure 1, right, **T9**, C and D), while in the ^{13}C NMR spectra of **T9-1** and **CuAAC-9**″ (Figure 1, right, **T9-1** and **CuAAC-9**″), no alkyne signal appeared. From this kind of quantitative ^{13}C NMR, the polymerization degrees ($\overline{DP}$ in Table 1) of these polymers were determined to be close to the polymerization degree (X_n) calculated from the formula shown in Table 1. The greater difference in sample **T12** was probably due to the difficulty of quantitatively weighing of liquid monomer **6** (Table 1). All the thermal click polypeptides **T9**-**T12** indicated good solubility in strong polar solvents such as DMSO, dimethylformamide (DMF), and water.

The copper contents of polymers obtained by thermal click chemistry (**T9**) as well as CuAAC (**CuAAC-9**, **CuAAC-9**″, and **CuAAC-9**″) were characterized by XPS (Figure 2). It is obvious that **T9** contains no copper residual (Cu/C = 0:100) because there was no copper catalyst used in the polymerization process. However, in the case of the polymers obtained by CuAAC, copper residual was easily confirmed, especially in sample **CuAAC-9′**, a Cu/C of 0.77:99.23 could be identified. We have mentioned that residual Cu ions in the products would have multiple toxic effects, especially when used in biomedical products involving proteins or cells. In this respect, these results show that polymers obtained by thermal click reaction are superior to ones obtained by CuAAC. This conclusion is also verified by the cell cytotoxicity assessment of **T9**-**T12**. The cytotoxicity of **T9**-**T12** was evaluated against L929 mouse fibroblasts in vitro by MTT assay. Chemically synthesized

Table 1. Characteristics of click polypeptides **T9-T12**, **CuAAC-9**, and click polypeptides with terminated mPEG (**mPx-r-T9-y**) or randomly distributed PEG (**Px-r-T9-y**).

Sample	Solubility[a)]	r[b)]	X_n[c)]	$\overline{DP}$ (NMR)	[1,4]/[1,5][d)]
T9	W, S, F	0.98	99	88.6[e)]	1.74/1.0
T10	W, S, F	0.98	99	89.5[e)]	1.79/1.0
T11	W, S, F	0.98	99	94.2[e)]	1.58/1.0
T12	W, S, F	0.98	99	68.9[e)]	1.76/1.0
mP1-T9-1	W, S	0.98	99	117.2[f)]	1.70/1.0
mP1-T9-2	W, S	0.94	32.3	29.9[f)]	1.59/1.0
mP1-T9-3	W, S	0.90	19	21.9[f)]	1.44/1.0
mP2-T9-1	W, S	0.98	99	120.6[f)]	1.66/1.0
mP2-T9-2	W, S	0.94	32.3	37.4[f)]	1.55/1.0
mP2-T9-3	W, S	0.90	19	23.8[f)]	1.57/1.0
mP5-T9-1	W, S	0.98	99	101.8[f)]	1.52/1.0
mP5-T9-2	W, S	0.94	32.3	35.7[f)]	1.48/1.0
mP5-T9-3	W, S	0.90	19	9.5[f)]	1.61/1.0
P1-*r*-T9-1	W, S	0.98	99	105.6[f)]	1.46/1.0
P1-*r*-T9-2	W, S	0.94	32.3	36.9[e)]	1.57/1.0
P1-*r*-T9-3	W, S	0.90	19	19.0[e)]	1.55/1.0
P2-*r*-T9-1	W, S	0.98	99	98.6[e)]	1.64/1.0
P2-*r*-T9-2	W, S	0.94	32.3	30.6[e)]	1.54/1.0
P2-*r*-T9-3	W, S	0.90	19	20.8[e)]	1.47/1.0
CuAAC-9	W, S, F	1	–	–	100/0

[a)]W = water, S = DMSO, F = DMF; [b)]r is the monomer ratio, $r = N_B/N_A$, $N_B/(N_A + 2N_{A'})$ or $N_B/(N_A + N_{A''})$, as shown in Scheme 2; [c)]Calculated according to $X_n = 1 + r/1 + r - 2rp$, which by assuming that the reaction was complete ($p = 1$) was converted to $X_n = 1 + r/1 - r$; [d)]The molar ratio of 1,4-triazole to 1,5-triazole as determined from ^{1}H NMR; [e)]From quantitative ^{13}C NMR (calculated by the comparison between characteristic peaks of terminal alkynes and triazoles, e.g., A, A′, B, B′, and A, D in Figure 1 [right]); [f)]From ^{1}H NMR.

Table 2. The numbers of PEG repeating units compared with that of amino groups of different polycations and the amino-group contents of microspheres immobilized with different polycations.

Polycation	PEG repeating units per amino group	Amino-group content of microspheres [μmol g^{-1}]
e-PL	0	37.1
PLL	0	47.7
T9	0	44.7
mP1-T9-1	0.191	46.2
mP1-T9-2	0.749	56.7
mP1-T9-3	1.022	35.6
mP2-T9-1	0.374	43.2
mP2-T9-2	1.206	38.6
mP2-T9-3	1.896	37.1
mP5-T9-1	1.113	43.2
mP5-T9-2	3.174	38.6
mP5-T9-3	11.926	37.1
P1-*r*-T9-1	0.517	35.6
P1-*r*-T9-2	1.204	35.6
P1-*r*-T9-3	2.152	40.2
P2-*r*-T9-1	1.253	35.1
P2-*r*-T9-2	3.035	34.1
P2-*r*-T9-3	4.487	31.0

PLL and biosynthesized ε-PL were used as the negative and positive control, respectively. As shown in Figure 3, without copper residual, **T9**-**T12** all exhibited much better cell compatibility than both ε-PL and PLL in all the three different concentrations (Figure 3). More visual results could be seen from the optical images of L929 fibroblast cells after incubated for 24 and 48 h (Figure 4). In the PLL system, most of the cells died within 24 h, and the remained living cells were smaller than 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} **T9**, **T10**, **T11**, or **T12** showed proliferation status during 48 h which was even better than that of ε-PL and close to blank control, indicating high cytocompatibility of the thermal click polypeptides systems.

3.3. PEGylated Click Polypeptides (PEG-CPs)

Since the cell cytotoxicity properties of thermal click polypeptides **T9**-**T12** are close, we simply chose **T9** as the representative to be PEGylated. Different kinds of click polypeptides with terminated mPEG (**mPEG-CP**s) and random-distributed PEG (**PEG-*r*-CP**s) were obtained by the copolymerization of monomer **3**, monomer **5** with alkyne-terminated mPEG and dialkyne-terminated PEG, respectively [Scheme 2(c) and Table 1]. For each alkyne-terminated mPEG or dialkyne-terminated PEG with different molecular weights (the molecular weights of mPEGs are 1000 [**1a**], 2000 [**1b**], and 5000 [**1c**], respectively; the molecular weights of PEGs are 1000 [**2a**] and 2000 [**2b**], respectively), **PEG-CP**s with three different monomer ratios ($r = 0.98$, 0.94, and 0.90, respectively, Table 1) were finally synthesized. These **PEG-CP**s were characterized by ^{1}H and ^{13}C NMR. $\overline{DP}$s of click polypeptides with terminated mPEG were determined by ^{1}H NMR. In the case of the click polypeptides with random-distributed PEG, since the signal of terminated alkyne groups could not be found in ^{1}H NMR (Table 1 and Figure 5), $\overline{DP}$s of these polymers were determined by quantitative ^{13}C NMR (Table 1 and Figure 6).

As an example, ^{1}H and ^{13}C NMR spectra of click polypeptide with terminated mPEG **mP5-T9-1** are presented in Figure 5. The appearance of the characteristic signal of triazole rings in ^{1}H NMR (Figure 5, left, a, a′), as well as ^{13}C NMR spectra (Figure 5, right, A, A′ and B, B′) indicated the successful procession of click reactions between dialkyne and diazide monomers. The signal of mPEG at $\delta = 3.64$ in ^{1}H NMR (Figure 5, left, c) and $\delta = 69.1$ in ^{13}C NMR spectra (Figure 5, right, C) as well as the disappearance of the signals of terminal alkyne groups of alkyne-terminated mPEG indicated the successful termination by alkyne-terminated mPEG. According to our design, for every polymer chain, there are two terminated mPEGs, so the $\overline{DP}$s of click polypeptides with terminated mPEG can be calculated by ^{1}H NMR showing in Table 1. The results are close to theoretical polymerization degrees (X_ns in Table 1). The ^{1}H and ^{13}C NMR spectra of click polypeptide with random-distributed PEG **P1-*r*-T9-1** are presented in Figure 6. The signals corresponding to PEG exist in ^{1}H (Figure 6, left, c) and ^{13}C (Figure 6, right, C) NMR spectra. Since the PEG segments are randomly distributed in the main chain, the number of PEG chains in each polymer chain is not clear. The polymers do have two alkyne groups in the chain terminals, but the signals of terminated alkyne groups could not be seen in ^{1}H NMR. However, in the quantitative ^{13}C NMR of **P1-*r*-T9-1** (Figure 6, right), the signals of terminated alkyne groups could be found (Figure 6, right, E and D), which were used to calculate the polymerization degree of this kind of polymers. The results are shown in Table 1, which are also close to theoretical polymerization degrees (X_ns in Table 1). The numbers of PEG repeating units comparing with that of amino groups of the two kinds of **PEG-CP**s are shown in Table 2.

For the study of ET removal, the two kinds of **PEG-CP**s were immobilized onto crosslinked PS-AE through the reaction between amino groups and activated ester groups[41] (Scheme 1). The amino group contents of these

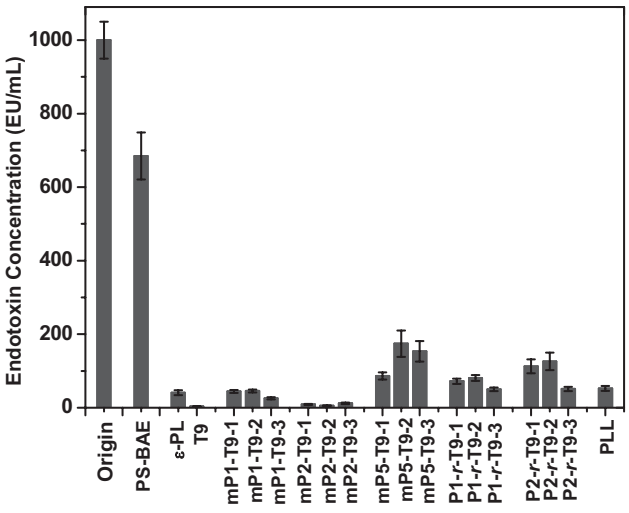


Figure 7. The change of ET concentration before (origin) and after treatment with different PS microspheres adsorbents [for 1 mL of ET solution (1000 EU · mL^{-1}), 10 mg microspheres was used].

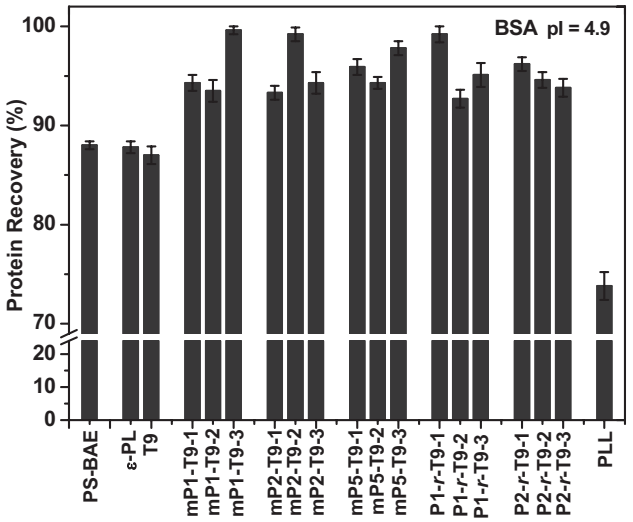


Figure 8. BSA recovery after treatment with **PS-BAE** and different polycations immobilized crosslinked PS microspheres [for 1 mL of BSA solution (0.488 mg · mL^{-1}), 10 mg microspheres was used].

microspheres were determined by ninhydrin technique according to literature[42] and controlled into a relatively narrow range (Table 2). ET containing solutions were then treated with these **PEG-CP**s immobilized microspheres, and the ET concentration remained in the solution decreased sharply from the initial 1000 EU · mL^{-1} to less than 200 EU · mL^{-1}, some of them even down to near zero (Figure 7), which are close to that of ε-PL or PLL immobilized microspheres. It was noteworthy that the unmodified blank microspheres with blocked activated ester groups (PS-BAE) also shows some ET absorbing capacity for its hydrophobic properties. It is interesting that, the click polypeptide without PEG segments (like **T9**) shows highest ET absorbing capacity, comparing with that of click peptides with higher PEG contents (like **P2-*r*-T9-2**) or longer PEG chains (like **mP5-T9-3**). The existence of PEG segments may also exhibit ET resistance to some extent. From these results, we can infer that, the PEG segments in polypeptides are not the longer the better or the more the better. Considering about the later protein recovery capacity, an optimum value may be required.

The most representative acidic protein–BSA was chosen to treat with the adsorbents to take the protein recovery experiments. As shown in Figure 8, **T9** immobilized microspheres had similar BSA recovery capacity (nearly 90%) to that of the blank PS-BAE microspheres and ε-PL immobilized microspheres, but much higher than that of PLL immobilized microspheres (75%). When the PEG segments were involved into the system, all the **PEG-CP**s immobilized microspheres showed higher BSA recoveries around 95%, and the difference between the two kinds of **PEG-CP**s was little (Figure 8). These results indicate that the protein recovery properties of the polycation immobilized microspheres could be significantly improved after PEGylation.

In order to further confirm the selective removal properties, **PEG-CP**s immobilized microspheres were directly treated with the ET mixed BSA solution. As shown in Figure 9, after treatment by polycation (ε-PL, **T9**, **PEG-CP**s, or PLL) immobilized microspheres, the ET concentration remaining in the mixture solution decreased vastly from the initial 11 500 EU · mL^{-1} to less than 1000 EU · mL^{-1}, some of them even decreased to 100 EU · mL^{-1}. Again, **T9** immobilized microspheres exhibited the best adsorption

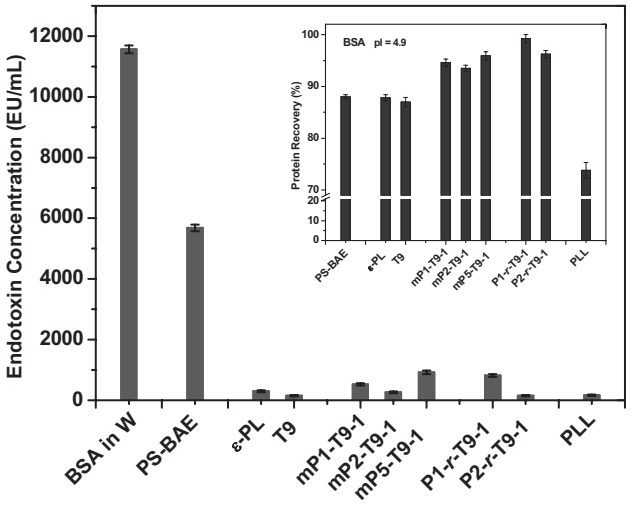


Figure 9. The ET adsorption and BSA recovery properties of ET containing BSA solution treated with **PS-BAE** and different polycations immobilized crosslinked PS microspheres. The concentrations of BSA and ET were 0.488 mg · mL^{-1} and 11 565 EU · mL^{-1}, respectively.

performance, with a high ET adsorption capacity of $1.14 \times 10^6\,EU \cdot g^{-1}$, which is the combined contribution of higher contents of amino groups and larger specific surface area of the microspheres. The ET adsorbing capacity of **PEG-CP**s immobilized microspheres is somewhat lower than that of **T9**, ε-PL, and PLL immobilized microspheres, indicating the weak ET resistance from PEG segments again. At the same time, the protein recoveries results of all the adsorbents (Figure 9, top right corner) were consistent with the results obtained by protein recovery experiments from pure BSA solution. **PEG-CP**s immobilized microspheres showed the highest BSA recoveries around 95% from the ET mixed solution. It is believed that trace amount of the ET in the protein solution could be removed easily and completely without much loss of the protein content by treating with **PEG-CP**s immobilized microspheres repeatedly.

4. Conclusion

A series of copper-free PEGlyated click polypeptides with low pK_a were realized by microwave-assisted thermal click polymerization with improved reaction efficiency for the first time. The low pK_a endowed the click polypeptides with little interaction with proteins, and the PEG segments provided excellent protein resistance ability. This form of PEGylated polycations exhibited high ET adsorbing ability and high protein recovery properties simultaneously, which can be used as ligands for ET selective removal from protein (especially acidic protein such as BSA) solution.

Acknowledgements: The authors would like to thank the financial support from National Natural Science Foundation of China (No. 20774094, 51021003, 50733003, and 20874097), the Ministry of Science and Technology of China (973 Project, No. 2009CB930102), "100 Talents Program" of the Chinese Academy of Sciences (No. KGCX2-YW-802), and Jilin Provincial Science and Technology Department (No. 20082104, 20100588).

Received: October 7, 2011; Revised: November 22, 2011; Published online: January 25, 2012; DOI: 10.1002/mabi.201100394

Keywords: biological applications of polymers; endotoxin removal; PEGylation; step-growth polymerization; thermal click chemistry

[1] M. Sakata, C. Todokoro, C. Hirayama, *Am. Biotechnol. Lab.* **2002**, *20*, 36.
[2] C. Hirayama, M. Sakata, *J. Chromatogr. B* **2002**, *781*, 419.
[3] T. E. Karplus, R. J. Ulevitch, C. B. Wilson, *J. Immunol. Methods* **1987**, *105*, 211.
[4] H. Matsumae, S. Minobe, K. Kindan, T. Watanabe, T. Tosa, *Biotechnol. Appl. Biochem.* **1990**, *12*, 129.
[5] H. Fang, J. Wei, Y. T. Yu, *Biomaterials* **2004**, *25*, 5433.
[6] Z. Yuan, M. Yu, J. H. Li, G. H. Hou, H. Y. Wang, *Biomaterials* **2005**, *26*, 2741.
[7] C. B. Li, S. Hein, K. Wang, *Mater. Sci. Technol.* **2008**, *24*, 1088.
[8] J. S. Guo, Y. Wei, D. F. Zhou, P. Q. Cai, X. B. Jing, X.-S. Chen, Y. B. Huang, *Biomacromolecules* **2011**, *12*, 737.
[9] V. V. Rostoytsey, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2002**, *41*, 2596.
[10] a) T. H. Li, X. B. Jing, Y. B. Huang, *Macromol. BioSci.* **2011**, *11*, 865; b) J. S. Guo, Y. B. Huang, X. B. Jing, X. S. Chen, *Polymer* **2009**, *50*, 2847.
[11] C. Barner-Kowollik, F. E. Du Prez, P. Espeel, C. J. Hawker, T. Jinkers, H. Schlaad, W. Van Camp, *Angew. Chem. Int. Ed.* **2011**, *50*, 60.
[12] a) Q. Shi, X. S. Chen, T. C. Lu, X. B. Jing, *Biomaterials* **2008**, *29*, 1118; b) Y. D. Han, Q. Shi, J. L. Hu, Q. Du, X. S. Chen, X. B. Jing, *Macromol. Biosci.* **2008**, *8*, 638.
[13] a) Q. Shi, Y. B. Huang, X. S. Chen, M. Wu, J. Sun, X. B. Jing, *Biomaterials* **2009**, *28*, 5077; b) T. H. Li, X. B. Jing, Y. B. Huang, *Polym. Adv. Technol.* **2011**, *22*, 1266.
[14] a) L. Wong, F. Khan, J. Micklefield, *Chem. Rev.* **2009**, *109*, 4025; b) M. Hetzer, G. J. Chen, C. Barner-Kowollik, M. H. Stenzel, *Macromol. Biosci.* **2010**, *10*, 119.
[15] J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 16793.
[16] J. C. Jewett, E. M. Sletten, C. R. Bertozzi, *J. Am. Chem. Soc.* **2010**, *132*, 3688.
[17] F. Gonzaga, G. Yu, M. A. Brook, *Macromolecules* **2009**, *42*, 9220.
[18] C. R. Becer, R. Hoogenboom, U. S. Schubert, *Angew. Chem. Int. Ed.* **2009**, *48*, 4900.
[19] I. Singh, Z. Zarafshani, J.-F. Lutz, F. Heaney, *Macromolecules* **2009**, *42*, 5411.
[20] N. K. Devaraj, R. Upadhyay, J. B. Haun, S. A. Hilderbrand, R. Weissleder, *Angew. Chem. Int. Ed.* **2009**, *48*, 7013.
[21] H.-S. Han, N. K. Devaraj, J. Lee, S. A. Hilderbrand, R. Weissleder, M. G. Bawendi, *J. Am. Chem. Soc.* **2010**, *132*, 7838.
[22] K. L. Kicck, E. Saxon, D. A. Tirrell, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 19.
[23] H. L. Zhang, Y. Ma, X.-L. Sun, *Chem. Commun.* **2009**, *45*, 3032.
[24] B. D. Mather, K. Viswanathan, K. M. Miller, T. E. Long, *Prog. Polym. Sci.* **2006**, *31*, 487.
[25] C. E. Hoyle, C. N. Bowman, *Angew. Chem. Int. Ed.* **2010**, *49*, 1540.
[26] a) M. Lomba, L. Oriol, R. Alcalá, C. Sánchez, M. Moros, V. Grazú, J. L. Serrano, J. M. De la Fuente, *Macromol. Biosci.* **2011**, *11*, 1505; b) V. T. Huynh, G. Chen, P. de Souza, M. H. Stenzel, *Biomacromolecules* **2011**, *12*, 1738.
[27] B. H. Lipshutz, B. R. Taft, *Angew. Chem., Int. Ed.* **2006**, *45*, 8235.
[28] K. Yamaguchi, T. Oishi, T. Katayama, N. Mizuno, *Chem. Eur. J.* **2009**, *15*, 10464.
[29] L. Bonami, W. Van Camp, D. Van Rijckegem, F. E. Du Prez, *Macromol. Rapid Commun.* **2009**, *30*, 34.
[30] M. Lammens, J. Skey, S. Wallyn, R. O'Reilly, F. Du Prez, *Chem. Commun.* **2010**, *46*, 8719.
[31] J. Y. Kim, J. C. Park, H. Kang, H. Song, K. H. Park, *Chem. Commun.* **2010**, *46*, 439.
[32] S. K. Yousuf, D. Mukherjee, B. Singh, S. Maity, S. C. Taneja, *Green Chem.* **2010**, *12*, 1568.
[33] J. Hong, Q. Luo, B. K. Shah, *Biomacromolecules* **2010**, *11*, 2960.

[34] C. Besset, S. Binauld, M. Ibert, P. Fuertes, J.-P. Pascault, E. Fleury, J. Bernard, E. Drockenmuller, *Macromolecules* **2010**, *43*, 17.
[35] A. Hucknall, S. Rangarajan, A. Chilkotiucknall, *Adv. Mater.* **2009**, *21*, 1.
[36] R. Barbey, L. Lavanant, D. Paripovic, N. Schüwer, C. Sugnaux, S. Tugulu, H.-A. Klok, *Chem. Rev.* **2009**, *109*, 5437.
[37] W. Agut, D. Taton, S. Lecommandoux, *Macromolecules* **2007**, *40*, 5653.
[38] B. Carbini, A. Benalil, M. Vaultier, *J. Org. Chem.* **1993**, *58*, 3736.
[39] A. Deiters, T. A. Cropp, M. Mukherji, J. W. Chin, J. C. Anderson, P. G. Schultz, *J. Am. Chem. Soc.* **2003**, *125*, 11782.
[40] W. H. Binder, R. Sachsenhofer, *Macromol. Rapid Commun.* **2008**, *29*, 1097.
[41] J. Hwang, R. C. Li, H. D. Maynard, *J. Controlled Release* **2007**, *122*, 279.
[42] J. Knott, V. Rossbach, *Angew. Makromol. Chem.* **1980**, *86*, 203.
[43] C. Besset, J. Bernard, E. Fleury, J.-P. Pascault, P. Cassagnau, E. Drockenmuller, R. J. J. Williams, *Macromolecules* **2010**, *43*, 5672.