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Synthesis of Polypeptide-Polyether Conjugates from an Activated Urethane Derivative of γ-Benzyl-L-glutamate as a Monomer

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Summary

N-(4-Nitrophenoxycarbonyl)-γ-benzyl-L-glutamate (**1**) was heated at 60 ºC in *N*,*N*-dimethylacetoamide (DMAc) in the presence of *p*-(*tert*-butyl)phenylmethylamine (**2**) to afford poly(γ-benzyl-L-glutamate) in high yield. ^{1}H NMR analysis of the obtained polymer revealed that the *p*-(*tert*-butyl)phenylmethyl group was successfully introduced into the polymer chain end. Under the same conditions, polycondensations of **1** in the presence of poly(ethylene glycol)s having amino terminals were performed. Both of the components were consumed quantitatively to afford the corresponding block copolymers having poly(γ-benzyl-L-glutamate) and poly(ethylene glycol) segments. The molecular weights of the copolymers were controlled by the feed ratio $[\mathbf{1}]_0$/[amino group in the macroinitiator]$_0$.

Introduction

Block copolymers containing polypeptide-segments have attracted much attention because the unique 3-dimensional structures of polypeptides such as α-helix and β-sheet would permit us to assemble the polymers into supramolecular organizations. α-Helical polypeptides such as poly(γ-benzyl-L-glutamate) are attractive polymeric building block with a rigid rod-like structure, of which covalent connections with random-coil polymer segments affords a variety of copolymers that would exhibit specific morphologies driven by the incompatibility between the segments [1-4]. Another interesting topic is fabrication of bio-active and bio-compatible materials by functionalization of hydrophilic polymers with polypeptide segments [5-7]. Besides these block copolymers, other polymer architectures such as graft copolymers [8-11] and star-shaped polymers [12] are also current focuses with large expectations for their unique properties.

To date, these polymer architectures having well-defined polypeptide segments have been constructed with utilization of the living ring-opening polymerization of amino acid *N*-carboxyanhydrides (NCAs) [13-15]. The most important advantage of this polymerization as a tool for polypeptide synthesis is that various primary and secondary amines can initiate the polymerization and consequently their residues can be incorporated into the chain end of the resulting polypeptide. This nature leads to successful applications of various polymers having amino groups in their terminals and side chains as macroinitiators.

Despite these advantages of the living polymerization of NCA, the susceptive nature of NCA to moisture and heat has been the obstacle for its practical applications. Therefore, development of other easily accessible monomers for polypeptide synthesis has been of great interest of the researchers in this field. Buess *et al*. used α-carboxy hydroxamic acid for polypeptide synthesis [16]. By heating under basic conditions, this compound underwent rearrangement into isocyanato carboxylic acid, which was a potential bifunctional monomer capable of giving the corresponding polypeptide. Orgel *et al*. reported the application of a highly activated urethane derivative of α-amino acids, *N*-imidazolylcarbonylamino acid, to the synthesis of cyclic oligopeptides [17-18]. This α-amino acid-derivative underwent intramolecular cyclization with releasing imidazole, and the resulting NCA underwent ring-opening polymerization. Kricheldorf *et al*. used a more stable compound, α-(*N*-aryloxy-carbonyl)amino-*ω*-carboxylalkane [19]. The aryloxycarbonyl group underwent thermal dissociation into the corresponding phenol and α-isocyanato-*ω*-carboxylic acid, of which polyaddition gave the corresponding polypeptide efficiently. However, this method was not effective for polypeptide synthesis from α-amino acid derivative.

Recently, we have succeeded in synthesizing poly(γ-benzyl-L-glutamate) (=poly(BLG)) by using *N*-(4-nitrophenoxycarbonyl)-γ-benzyl-L-glutamate (**1**) as a monomer [20]. By heating **1** in aprotic polar solvents such as *N*,*N*-dimethyl-formamide and *N*,*N*-dimethylacetamide, poly(BLG) was obtained efficiently. The obtained poly(BLG) had a number average molecular weight (M_n) higher than 15,000 and exhibited virtually same chiro-optical profile as that of the poly(BLG) obtained by the ring-opening polymerization of NCA of γ-benzyl-L-glutamate. An important phenomenon observed therein was that **1** underwent intramolecular cyclization to give the corresponding NCA in the initial stage of the polymerization. This finding made us speculate that the predominant pathway for the formation of poly(BLG) would involve the ring-opening polymerization of NCA. Based on this speculation, we expected that primary amine-type components could be combined with the system as potential initiators for the polymerization of NCA to obtain polypeptides having the residues of the amines at the terminals. Herein, we report polycondensations of **1** in the presence of primary amines, which included amine-terminated poly(ethylene glycol)s as macroinitiators for the synthesis of polyether-polypeptide conjugates.

Experimental

Materials

N-(4-nitrophenoxycarbonyl)-γ-benzyl-L-glutamate (**1**) was synthesized according to the previously reported method [20]. Poly(ethylene glycol) methyl ether (typical M_n 2,000) and poly(ethylene glycol) bis(3-aminopropyl) terminated **6** (typical M_n 2,000) were purchased from SIGMA-ALDRICH, Inc. (St. Louis, USA). *N*,*N*-dimethyl-

acetoamide (DMAc) used for the polycondensation was distilled over calcium hydride prior to use. The other solvents were purchased from Wako Pure Chemical Indusries Ltd. (Osaka, Japan) and used as received. The amination rate of the mono-amino-terminated poly(ethylene glycol) and poly(ethylene glycol) bis (3-aminopropyl) terminated was analyzed by MALDI-TOF mass spectroscopy.

Measurements

^{1}H (400 MHz) and ^{13}C NMR (100.6 MHz) spectra were recorded on a Varian NMR spectrometer model Unity INOVA, using tetramethylsilane (TMS) as an internal standard in $CDCl_3$. Fourier-transfer infrared spectroscopy (FT-IR) was performed using a JASCO FTIR 460 plus spectrometer. Number-average molecular weight (M_n) and weight-average molecular weight (M_w) were estimated by size-exclusion chromatography (SEC) on a TOSOH HLC-8220 system equipped with three consecutive polystyrene gel columns [TSK-gels (bead size, exclusion limited molecular weight); super-AW4000 (6 μm, $> 4 \times 10^5$), super-AW3000 (4 μm, $> 6 \times 10^4$), and super-AW2500 (4 μm, $> 2 \times 10^3$)] and refractive index and ultraviolet detectors at 40 ºC. The system was operated at a flow rate of 0.5 mL/min, using *N*,*N*-dimethylformamide (DMF) solution including lithium bromide (10 mM) as an eluent. Polystyrene standards were employed for calibration. MALDI-TOF mass was carried out on a PerSeptive Biosystems Voyager DE Pro Bio Spectrometry workstation. Measurement condition was described in Electronic Supplementary Information.

*Synthesis of the mono amine-type macroinitiator **5***

Mono amine-terminated poly(ethylene glycol) was prepared from poly(ethylene glycol) methyl ester (10.0g, 50.0 mmol) by using our method [21]. Mono amine-terminated poly(ethylene glycol) was obtained in 70% yield (7.0 g, 35.0 mmol) as white compound. ^{1}H NMR ($CDCl_3$): δ= 2.03 (br, 2H, -CH_2-NH_2), 1.65-2.37 (br, 5H, -O-CH_3 and –O-CH_2-CH_2-), 4.22 (t, 2H, J = 5.2 Hz, -CH_2-O-C(=O)-), 5.68 (br, 1H, -C(=O)-O-N*H*-); ^{13}C NMR ($CDCl_3$): δ= 58.8 (-HN-*C*H_2-CH_2-NH_2), 61.5 (-NH-CH_2-*C*H_2-NH_2), 67.7 (-O-*C*H_3), 70.5 (-O-*C*H_2-CH_2-O-), 71.8 (-*C*H_2-O-CH_3), 161.3 (-*C*(=O)-NH-); FT-IR (KBr): 2887, 1541, 1472, 1343, 1281, 1113, 1061 cm^{-1}.

*Synthesis of the polypeptide **4***

A solution of **1** (0.40 g, 1.0 mmol) and 4-(*tert*-butyl)phenylmethylamine (35.0 μL; 3.3 mg, 0.2 mmol) in *N*,*N*-dimethylacetamide (DMAc) (1.0 mL) was stirred at 60 ºC for 48 h under nitrogen. The reaction mixture was poured into ether (200 mL), and the resulting precipitate was collected by filtration with suction and dried under vacuum to obtain **4** a whitish brown solid in 73 % yield: ^{1}H NMR ($CDCl_3$): δ= 1.25 (m, 3H×3, -CH-CH_3), 1.72-2.82 (br, 2H, >CH-CH_2-CH_2- and br, 2H, >CH-CH_2-CH_2-), 3.81-4.12 (br, >C*H*-CH_2-CH_2), 4.16-4.46 (-NH-CH_2-C_6H_4-), 5.02 (br, 2H, -C(=O)-O-CH_2-), 7.26 (br, 5H, -O-C_6H_5 and br, 4H, -CH_2-C_6H_4-C-$(CH_3)_3$).

*Synthesis of the AB diblock copolymers **7***

A solution of **1** (0.40 g, 1.0 mmol) and the monoamine-terminated PEG **5** (0.04 g, 0.02 mmol) in *N*,*N*-dimethylacetamide (DMAc) (1.0 mL) was stirred at 60 ºC for 48 h

under nitrogen. The reaction mixture was poured into ether (200 mL), and the resulting precipitate was collected by filtration with suction and dried under vacuum to obtain the AB block copolymer **7** as a whitish brown solid in quantitative yield: ^{1}H NMR ($CDCl_3$): δ= 1.58-2.85 (br, 2H, >CH-CH_2-CH$_2$- and br, 2H, >CH-CH$_2$-CH_2-), 2.89-3.16 (br, 3H, -O-CH_3), 3.65 (br, 2H, -O-CH_2-CH$_2$-O-, and br, 2H, -O-CH$_2$-CH_2-O-), 3.94 (br, 1H, >C*H*-CH$_2$-), (br, 1H, -CH_2-C$_6$H$_5$), 6.44 (s, 1H, –C(=O)-N*H*-CH$_2$- and s, 1H, >CH-N*H*-C(=O)-), 7.27 (br, 5H, -CH$_2$-C$_6H_5$); ^{13}C NMR ($CDCl_3$): δ= 21.3 (>CH-*C*H$_2$-CH$_2$-), 28.0 (>CH-CH$_2$-*C*H$_2$-), 30.7 (-NH-*C*H$_2$-CH$_2$-NH-), 35.1 (-NH-CH$_2$-*C*H$_2$-NH-), 37.9 (-O-*C*H$_3$), 54.0 (>*C*H-CH$_2$-), 66.0 (-O-*C*H$_2$-CH$_2$-O-), 66.6 (-O-*C*H$_2$-CH$_2$-O-), 70.4 (-*C*H$_2$-C$_6$H$_5$), 125.9, 128.0, 128.2, 128.3, 128.4, 135.8 (-*C*$_6$H$_5$), 167.6 (-NH-*C*(=O)-O-CH$_2$-CH$_2$-), 171.9 (>CH-NH-*C*(=O)-O-), 172.7 (>CH-*C*(=O)-); FT-IR (KBr): 3293, 3034, 2880, 1734 ($\nu_{C=O}$ –C(=O)-O-), 1653 ($\nu_{C=O}$ –NH-C(=O)-O-), 1542, 1456, 1393, 1166 cm^{-1}.

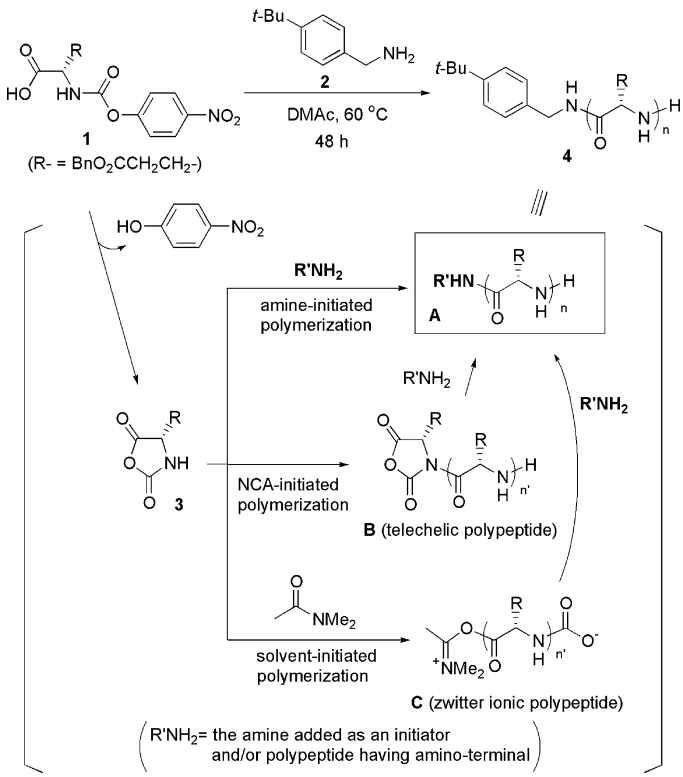
*Synthesis of ABA block copolymers **8***

A typical procedure: A solution of **1** (0.40 g, 1.0 mmol) and poly(ethylene glycol) bis (3-aminopropyl) terminated **6** (0.04 g, 0.02 mmol) in *N*,*N*-dimethylacetamide (DMAc) (1 mL) was stirred at 60 ºC for 48 h under nitrogen. The reaction mixture was poured into ether (200 mL), and the resulting precipitate was collected by filtration with suction and dried under vacuum to obtain the ABA block copolymer **8** as a whitish brown solid in 82 % yield: ^{1}H NMR ($CDCl_3$): δ= 1.95-2.79 (br, >CH-CH_2-CH$_2$- and >CH-CH$_2$-*CH*$_2$-), 3.66 (s, 2H, -O-CH_2-CH$_2$-O- and s, 2H, -O-CH$_2$-CH_2-O-), 3.76-3.94 (br, 1H, -NH-C*H*-C(=O)-), 4.09 (t, 1H, *J* = 5.6 Hz, oligomer -NH-C*H*-C(=O)-), 5.05 (br, 2H, -O-CH_2-C$_6$H$_5$), 7.27 (br, 5H, -CH$_2$-C$_6H_5$); ^{13}C NMR ($CDCl_3$): δ= 25.4 (>CH-*C*H$_2$-CH$_2$-), 29.8 (>CH-CH$_2$-*C*H$_2$-), 54.1 (>*C*H-), 66.0 (-O-*C*H$_2$-CH$_2$-O-), 66.6 (-O-CH$_2$-*C*H$_2$-O-), 70.4 (-*C*H$_2$-C$_6$H$_5$), 126.0, 128.0, 128.2, 128.4, 128.5, 135.3 (-*C*$_6$H$_5$), 171.9 (>CH-NH-*C*(=O)-O-), 172.8, (>CH-*C*(=O)-NH-); FT-IR (KBr) 3293, 2878 (ν_s CH_3 group and ν_{CH} CH_2 group), 2359, 1733 ($\nu_{C=O}$ –C(=O)-O-), 1653 ($\nu_{C=O}$ –NH-C(=O)-O-), 1541, 1507, 1456, 1165 cm^{-1}.

Results and Discussion

1 was synthesized by the carbamoylation reaction of γ-benzyl-L-glutamate with 4-nitrophenyl chloroformate. Previously, we have reported **1** reacted smoothly in its *N*,*N*-dimethylacetoamide (DMAc) solution (concentration=2.0 M) at 60 °C, to give poly(BLG) effectively. Based on this result, we attempted a polymerization of **1** in the presence of a primary amine under the same conditions (Scheme 1). As a primary amine, 4-(*tert*-butyl)phenylmethylamine (**2**) was chosen because the *tert*-butyl group would be a convenient probe in ^{1}H NMR analysis for confirming incorporation of the amine into the corresponding polymer. Besides this polymerization, that with using butylamine was also performed (Scheme S-1 in Electronic Supplementary Information).

We performed the polycondensation with a low monomer/initiator ratio to obtain poly(BLG) having a low molecular weight, which allowed us to analyze the chain end structure by ^{1}H NMR precisely. A solution of **1** and **2** in DMAc ([**1**]$_0$=2.0 M, [**1**]$_0$/[**2**]$_0$=100/20) was heated at 60 °C for 48 h, and the resulting compound was isolated as a diethyl ether-insoluble fraction in 73% yield. Size exclusion chromatography (SEC) of the product indicated a unimodal elution peak, for which

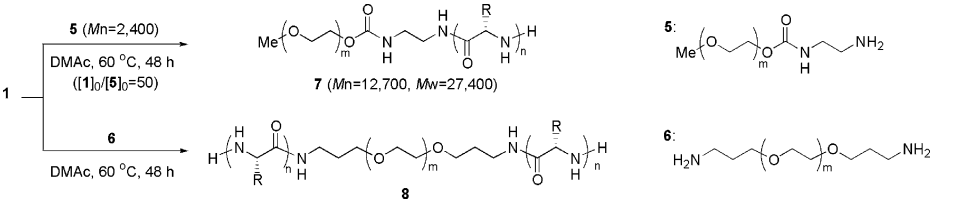


Scheme 1. Polycondensation of **1** in the presence of amine **2** and the corresponding reaction pathways.

M_n and M_w were estimated to be 1,000 and 1,500, respectively. The ^{1}H NMR spectrum of the obtained compound clearly showed the peaks of methine proton, and benzylic proton at 4.16-4.46, 5.02 ppm, respectively. A set of signals at 1.27 ppm and 1.68-2.80 ppm, which were assigned to the *t*-butyl moiety and the methylene moiety at benzylic position were observed (Figure S-1 in Electronic Supplementary Information). Another evidence for the successful incorporation of the amine into the chain end was obtained by MALDI-TOF mass analysis of the obtained poly(BLG) (Figure S-2 and Table S-1 in Electronic Supplementary Information). These moieties were inherited from the amine **2**, confirming that the amine was successfully incorporated into the chain end of the polymer **4**.

In our previous paper, we reported that the *in situ* monitoring of the polycondensation of **1** by ^{1}H NMR with using DMF-d_7 as a solvent revealed that a significant amount of NCA **3** was formed by the intramolecular cyclization of **1** and its ring-opening polymerization would be the predominant mechanism for the formation of poly(PLG) [20]. Similarly, the polycondensation of **1** in the presence of the amine **2** was monitored by ^{1}H NMR (Figure S-3 in Electronic Supplementary Information). Formation of **3** was clearly observed, allowing us to propose several possible pathways for the formation of the polypeptides from **3**. Other minor pathways such as intermolecular polycondensation of **1** would be also possible; however, they were not taken into account at present in order to simplify the discussion (Scheme S-2 in

Electronic Supplementary Information). One of the major pathways would be the amine-initiated ring-opening polymerization to give the corresponding polypeptide having amino terminal (=polypeptide A). Another possible mechanism would be initiation of the polymerization of NCA by NCA, which is discussed in the recent review by Kricheldorf [22]. This mechanism permits formation of a telechelic polypeptide (polypeptide B), which has an electrophilic NCA moiety at the initiating end and a nucleophilic amino moiety at the propagating end. In the present polymerization system with using **1**, the amine added as the second component can be immediately trapped by the NCA terminal of polypeptide B to give the polypeptide A having an amide group in the initiating end. One more important mechanism would involve the initiation of the ring-opening polymerization of **3** by the polarized solvent such as DMF and DMAc, which affords a zwitter ionic polypeptide C. Because this polypeptide has a highly electrophilic imminium moiety in the chain end, it would react with the amine with releasing DMAc. The successful incorporation of the monofunctional amine into the chain end of the polypeptide prompted us to apply amine-terminated polymers as a component for synthesizing block copolymers having polypeptide segments (Scheme 2). As such polymeric components, two terminal-functionalized poly(ethylene glycol)s **5** and **6** were chosen. **5** was synthesized from poly(ethylene glycol) mono-methyl ether and **6** was commercially available. The amination ratio of the mono-amino-terminated poly(ethylene glycol) and poly(ethylene glycol) bis (3-aminopropyl) terminated was analyzed by MALDI-TOF mass spectroscopy. The MALDI-TOF mass spectrum of the both macroinitiators confirmed that amine was incorporated into the chain end of the macroinitiators, completely.



Scheme 2. Syntheses of polyether-polypeptide conjugates.

First, a mono amine-type macroinitiator **5** (M_n = 2,400, M_w = 2,500) was employed to synthesize an AB-type diblock copolymer having one poly(ethylene glycol) (=PEG) segment and one polypeptide segment. A DMAc solution of **1** and **5**, of which feed ratio $[\mathbf{1}]_0/[\mathbf{5}]_0$ was 50, was heated at 60 ºC for 48 h. The mixture was poured into diethyl ether, and the resulting precipitates were collected by filtration. SEC analysis of the product indicated a unimodal elution peak, for which M_n and M_w were estimated to be 12,700 and 27,400, respectively (Figure 1). The elution peak was clearly distinguished from that for **5**, to suggest that the amine terminal of **5** was successfully incorporated into the corresponding AB-type block copolymer **7** as a junction between the two segments. Both of the polyether and polypeptide segments were clearly observed in the ^{1}H NMR spectrum (Figure S-4 in Electronic Supplementary Information).

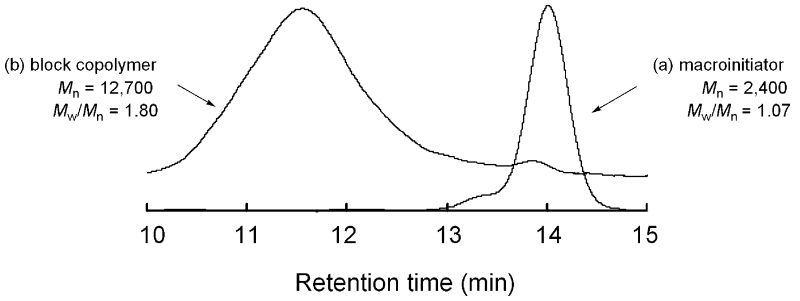


Figure 1. SEC profiles (detected by RI) for (a) the macroinitiator **5** and (b) the obtained block copolymer **7**.

Next, with using a bis-amine type macroinitiator **6** (M_n = 2,000, M_w = 2,600), synthesis of an ABA-type triblock copolymer was attempted. When DMAc solution of **1** and **6** was heated at 60 °C for 48 h ($[\mathbf{1}]_0/[\mathbf{6}]_0$=100/1), **1** was completely consumed to give the corresponding block copolymer **8** in 93% yield (isolated as a diethyl ether-insoluble fraction), of which SEC-estimated M_n and M_w were 15,400 and 23,300, respectively. The SEC profile confirmed that the polyether was completely incorporated into the triblock copolymer. Similarly to the case for the AB-type block copolymer **7**, the ^{1}H NMR spectrum of **8** indicated both of the polyether and polypeptide segments.

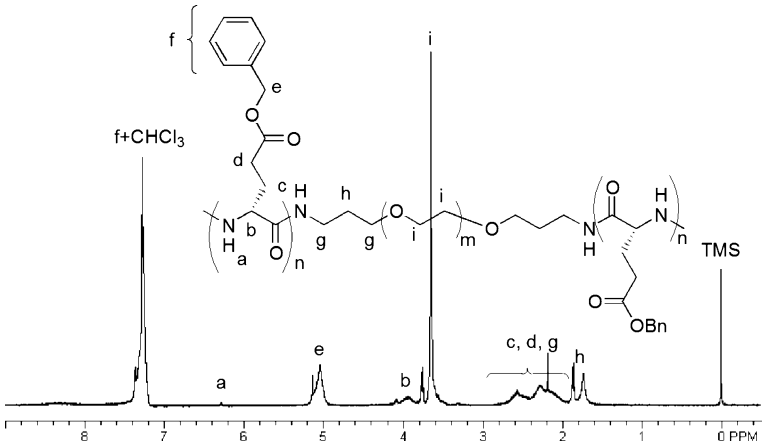


Figure 2. ^{1}H NMR spectrum of ABA block copolymer.

The mechanism for the successful formation of the copolymers **7** and **8** would be analogous to that for the polycondensation in the presence of the monofunctional amine **2**.

Finally, we performed a series of the polycondensations of **1** in the presence of **6**,with varying the feed ratio $[\mathbf{1}]_0/[\mathbf{6}]_0$ from 25 to 125. Figure 3 shows the dependences of M_n and M_w/M_n of the resulting copolymer on the feed ratio. M_n increased proportionally to the feed ratio, confirming that the present system would inherit one of the characteristics of the living ring-opening polymerization of NCA, *i.e.*, it is virtually

free from chain transfer and thus molecular weight of polypeptide can be controlled by monomer/initiator ratio.

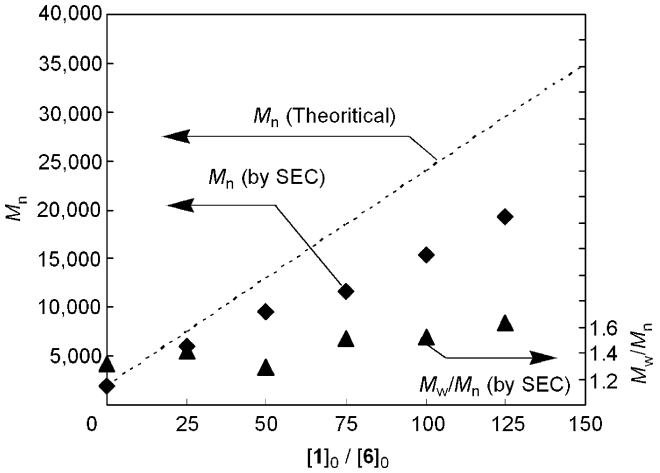


Figure 3. Dependence of M_n of the tri-block copolymer **8** on a feed ratio $[\mathbf{1}]_0/[$the macroinitiator **6**$]_0$.

Conclusion

In summary, a new synthetic approach to polypeptide-polyether conjugates has been achieved. The starting materials required for the present system were *N*-(4-nitrophenoxycarbonyl)-γ-benzyl-L-glutamate (**1**) and amine-terminated poly(ethylene glycol)s, and just heating their mixture in DMAc at 60 °C afforded the corresponding block copolymers efficiently. Further efforts to optimize the polycondensation conditions will be continued in order to sophisticate this highly facile and convenient method into a highly versatile and precise method for synthesis of a wide variety of block copolymers having polypeptide segments.

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