A New Strategy for Synthesis of ''Umbrella-Like'' Poly(ethylene glycol) with Monofunctional End Group for Bioconjugation

YANNAN ZHANG, GUOWEI WANG, JUNLIAN HUANG

The Key Laboratory of Molecular Engineering of Polymer, State Education Ministry of China, Department of Macromolecular Science, Fudan University, Shanghai 200433, People's Republic of China

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ABSTRACT: A novel strategy was used to synthesize poly(ethylene glycol) (PEG) with "umbrella-like" structure containing a single reactive group at the "handle" of the "umbrella". 1-(Bis(2-hydroxyethyl)amino)-3-(1-ethoxyethoxy)propan-2-ol was used to initiate the ring-opening polymerization (ROP) of ethylene oxide (EO) in the presence of diphenylmethylpotassium (DPMK) to obtain three-arm PEG (PEG3), then terminated by benzyl bromide or ethyl bromide. The resultant PEG3 was hydrolyzed to generate hydroxyl group at the conjunction point, and the second step ROP of EO was carried out using PEG3-OH as macroinitiator in the presence of DPMK. The obtained four-arm PEG (PEG4) contained a functional hydroxyl group at the end of the fourth arm, which could be easily modified to bioactive groups such as carboxyl, active ester, amino, etc. The well-defined structure of "umbrella-like" PEG was characterized by GPC, ^{1}H NMR, and MALDI-TOF MS in detail. Propionic acid succinimidyl ester of PEG4 (10 kDa) was utilized for protein conjugation with interferon α-2b. © 2010 Wiley Periodicals, Inc. J Polym Sci Part A: Polym Chem 48: 5974–5981, 2010

KEYWORDS: branched; poly(ethylene glycol); protein conjugation; ring-opening polymerization; star polymers; "umbrella-like" structure

INTRODUCTION As therapeutic agents, peptides and proteins are often rapidly cleared from circulation or give rise to immunological problems. To overcome these problems, many hydrophilic or amphiphilic polymers have been used to produce protein conjugation,[1,2] which could help proteins to increase stability toward proteolysis, as well as reduce renal excretion and immunological complications. Poly(ethylene glycol) (PEG) was the best candidate for protein modification due to its amphiphilicity, biocompatibility, low immunogenicity, and lack of toxicity.[3–7] The research on covalent coupling PEG to pharmaceutical proteins, commonly named PEGylation, was regarded as an extremely useful procedure to overcome certain problems faced in the development and application of protein drugs.[8–11] Up to now, many effective PEGylated proteins have appeared on the market.[12]

Linear monomethoxypoly(ethylene glycol) (mPEG) and heterotelechelic PEG derivatives were often used as PEGylation reagents.[8] Gilbert and Park-Cho[13] used linear mPEG (12 kDa) to develop PEG-interferon α-2b (IFN-α-2b) which enhanced pharmacokinetic properties in both animals and humans.[14] In 1994, development of first generation PEG-IFN-α-2b with once a week dosing was discontinued during Phase-II clinical trial because efficacy equivalent to IFN-α-2b given three times weekly was not achieved. In addition to the linear PEG derivatives, branched PEGs with single reactive functional group, as the second generation PEGylation reagents, have been proven more useful in protein and polypeptide modification.[15–17] One large PEG at a single site of protein was preferable to several small PEGs attached at multiple sites due to the "umbrella-like effect".[18] The branched PEG–protein conjugates exhibited increased pH and thermal stability, as well as greater stability toward proteolytic digestion.[16] Bailon et al.[19] conjugated IFN-α-2a with an *N*-hydroxysuccinimide (NHS) ester derivative of two-arm PEG (40 kDa), and the products showed improved pharmacokinetic properties in studies of animals and healthy humans.[20]

With the development of PEGylation technology, branched PEGs having a single reactive group, also named umbrella-like PEG, were need in large amount and high degree of purity. So many insights had been gained on the preparation of umbrella-like PEGs. The branched PEGs were normally prepared by an organic reaction between hydroxyl groups of linear PEG and functional branched agents such as trichloro-*s*-triazine or lysine,[15,21–26] making the synthesis procedure difficult and costly. These coupling approaches had a common disadvantage that the uncoupled linear PEGs had to be separated through complicated procedure. Taking the method linking mPEG succinimidyl carbonate (mPEG-SC) to lysine,[23] the reaction mixture contained impurities, which consisted of unreacted mPEG-SC, mPEG-OH (mPEG-SC would

Additional Supporting Information may be found in the online version of this article. Correspondence to: J. Huang (E-mail: jlhuang@fudan.edu.cn)
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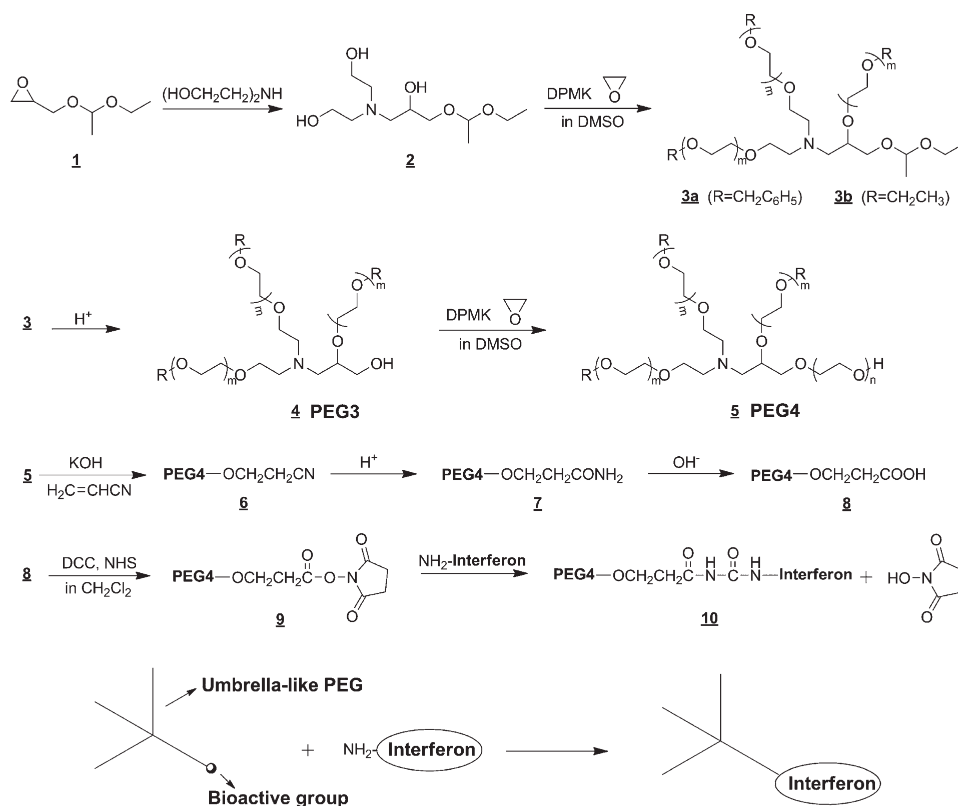

SCHEME 1 Synthesis of NHS-functionalized "umbrella-like" PEG and conjugation of resulting PEG to IFN-α-2b.

be very easily hydrolyzed to mPEG-OH in the moisture), lysine coupled with one PEG arm (PEG1), and lysine coupled with two PEG arm (PEG2). The products should be purified by time-consuming column chromatography separation procedure with low efficiency.

Following the development of anionic ring-opening polymerization (ROP) technology of ethylene oxide (EO), PEGs with various structure and functional groups may be easily resulted by some special designed initiator.[27,28] Therefore the purification problem in the branched PEG synthesis may be resolved by direct ROP of EO using a special designed initiator. But it was not easy to obtain branched PEG with monofunctional end group through ROP of EO due to the complicated synthesis procedure. Up to now, a variety of star-like and dendrimer-like PEGs with multifunctional groups at end of arms had been synthesized for drug or DNA delivery.[29,30] However, if these multifunctional branched PEG were used for protein modification, crosslinking in the system would be unavoidable[31] and that was why branched PEGs with monofunctional end group were required for protein modification since its monofunctionality would yield cleaner chemistry.

In this article, a novel synthetic route was established for "umbrella-like" PEG with a single bioactive functional group at the "handle" of the "umbrella" through two-step anionic ROP of EO. First, a small molecule compound 1-(bis(2-hydroxyethyl)amino)-3-(1-ethoxyethoxy)propan-2-ol (bHAEPO) and diphenylmethylpotassium (DPMK) were used as binary initiation system to get three-arm PEG (PEG3). The resultant PEG3 was hydrolyzed to generate hydroxyl group at the conjunction point, and then the second step ROP of EO was carried out using PEG3-OH as macroinitiator in the presence of DPMK. The obtained four-arm PEG (PEG4) had a functional hydroxyl group at the end of the fourth arm which could be converted to bioactive functional group by several transforming reactions.[32] Propionic acid succinimidyl ester of PEG4 (PEG4-SPA) (10 kDa) were used to react with INF-α-2b, resulting polymer–protein conjugation as summarized in Scheme 1.

RESULTS AND DISCUSSION

Synthesis of the Initiator (2) and PEG3 (3a and 4a)

Ethoxyethyl glycidyl ether (EEGE) (**1**) was synthesized through the reaction between glycidol and ethyl vinyl ether[33] with efficiency of 98% (see Supporting Information). The acetal protected hydroxyl group in EEGE did not result in side reactions in the polymerization of EO, and it could be easily hydrolyzed to generate another hydroxyl group. bHAEPO (**2**) possessing three hydroxyl groups and a protected one was obtained by reaction of purified EEGE with diethanolamine. In the ^{1}H NMR spectrum for bHAEPO [Fig. 2(A)], the characteristic resonance signals for methyl protons [—$CH_2C\boldsymbol{H_3}$, 1.19 ppm (*i*) and —$CHC\boldsymbol{H_3}$, 1.29 ppm (*h*)], methylene protons adjacent to nitrogen atom [—$C\boldsymbol{H_2}$—N—$(C\boldsymbol{H_2})_2$—, 2.61–2.75 ppm] (*c*, *d*), methylene protons [—$C\boldsymbol{H_2}CH_3$, —$C\boldsymbol{H_2}$—OH and —$C\boldsymbol{H_2}$—CH—OH, 3.55–3.66 ppm], methine proton [—C***H***—OH, 3.82 ppm], methine proton of acetal group [—OC***H***(CH_3)O—, 4.70 ppm] were all detected.

TABLE 1 Molecular Weight (MW) Data of PEG3 and PEG4

	PEG3 (**3a**)				PEG4 (**5a**)			PEG$_A$	PEG$_B$
	$M_{n(GPC)}$[a]	PDI[a]	$M_{n(NMR)}$[b]	$M_{n(MALDI\text{-}TOF\ MS)}$[c]	$M_{n(GPC)}$[a]	PDI[a]	$M_{n(MALDI\text{-}TOF\ MS)}$[c]	M_n[d]	M_n[e]
I	1,700	1.11	2,300	2,350	3,600	1.16	5,500	800	3,100
II	2,700	1.09	3,400	3,580	4,900	1.11	5,400	1,200	1,800
III	7,000	1.12	8,000	8,010	8,400	1.26	10,200	2,700	2,200

[a] Determined by GPC with 0.1 M aqueous $NaNO_3$ as solvent, calibrated by PEO standards.
[b] Determined from ^{1}H NMR ($CDCl_3$) by the end group analysis [Fig. 2(B)].
[c] Calculated by software from MALDI-TOF MS data.
[d] Obtained as one third of $M_{n(MALDI\text{-}TOF\ MS)}$ of PEG3 after rounding to two significant figures.
[e] The difference between $M_{n(MALDI\text{-}TOF\ MS)}$ of PEG3 and that of PEG4 after rounding to two significant figures.

PEG3 were grown from hydroxyl groups of bHAEPO (**2**) after partial deprotonated (30%) by DPMK. The proton exchange rate between hydroxyl groups of bHAEPO (**2**) and DPMK was faster than the propagation of EO,[34] so the molecular weight distribution of PEG3 was narrow, as shown in Table 1. After the polymerization finished, an additional DPMK solution was injected to the system to guarantee the complete deprotonation of all hydroxyl groups. Then excess benzyl bromide or bromoethane (2 equiv) was used to terminate living PEG chains. Benzyl-terminated PEG3 (**3a**) were preferable to be taken as example in the following steps due to its easy characterization. The synthesis and characterization of ethyl-terminated PEG3 (**3b**) were described in Supporting Information. From the gel permeation chromatographic (GPC) results of PEG3 (Fig. 1, solid line), the monomodal distribution curve indicated the successful polymerization of EO. However, the MW of **3a** determined from GPC (3000 Da) showed a clear deviation from the theoretical value ($M_{n(theory)}$ = 3600 Da, calculated by the concentration ratio of [initial monomer]/[initiator]). It could be attributed to the smaller hydrodynamic volume of branched PEG compared with linear PEG standard samples which were used as calibration in GPC.

^{1}H NMR spectrum for **3a** [Fig. 2(B)] also provided evidence for the successful synthesis of PEG3. The characteristic resonance signals for acetal proton [—OC***H***(CH_3)O—, 4.70 ppm] (*a*), methylene protons of PEG backbone (3.51–3.77 ppm) (*j*), methylene protons adjacent to nitrogen atom [—C$\boldsymbol{H_2}$—N—(C$\boldsymbol{H_2}$)$_2$—, 2.61–2.75 ppm] (*c*, *d*), phenyl group protons [—$CH_2C_6$$\boldsymbol{H_5}$, 7.33 ppm] (*l*) and methylene protons [—C$\boldsymbol{H_2}$-C_6H_5, 4.58 ppm] (*k*) were all detected. The calculated $M_{n(NMR)}$ of **3a** (sample II) was ~3400 Da, which was more approximate to $M_{n(theory)}$. The efficiency (*E*) of end-capping reaction at the end of polymerization of PEG3 can be calculated from Figure 2(B) by the following eq 1:

$$E = \frac{A_k/6}{A_a} \quad (1)$$

E values of the obtained samples (Table 1) were all above 98%.

MALDI-TOF MS analysis [Fig. 3(A)] was used to obtain a more reliable MW of PEG3. The major series of the molecular masses of PEG3 (**3a**) were expressed in the following equation: MW_{MS} = 44.05*n* (EO) + 248.30 ($C_{11}H_{22}NO_5$) + 273.39 (three benzyl groups) + 22.99 (sodium). The results strongly supported that the benzyl-terminated PEG3 were

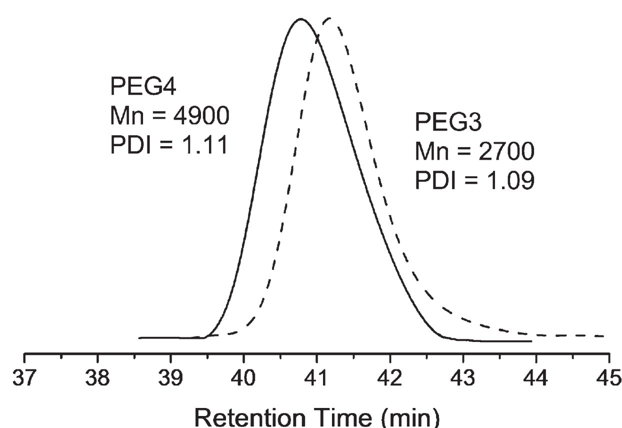

FIGURE 1 GPC traces of PEG3 (**3a**, dashed line) and PEG4 (**5a**, solid line) (Sample II in Table 1).

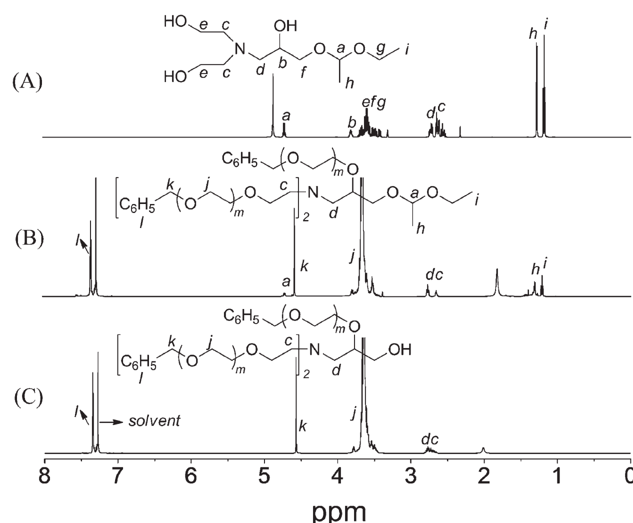

FIGURE 2 ^{1}H NMR spectra of bHAEPO (A), PEG3-OCH(CH_3)OC_2H_5 (B), and PEG3-OH (C) (Sample II in Table 1).

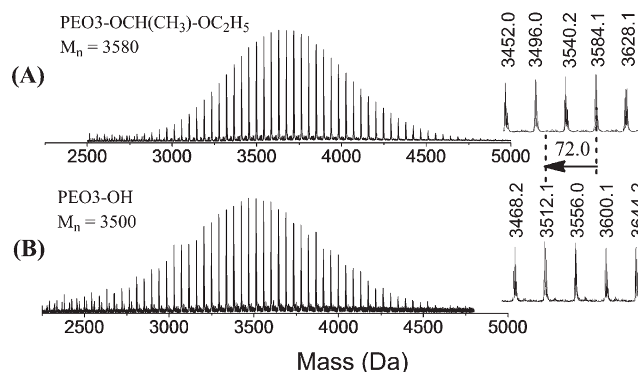

FIGURE 3 MALDI-TOF MS spectra of (A) PEG3-OCH(CH_3)OC_2H_5 (**3a**) and (B) PEG3-OH (**4a**) (Sample II in Table 1).

successfully prepared. The mass of **3a** appeared ~3580 Da, which was a good accordance with the theoretical value ($M_{n(theory)}$ = 3600 Da). In the following experimental procedure and discussion, $M_{n(MALDI\text{-}TOF\ MS)}$ was chosen as the exact value for calculation.

The acetal group at the conjunction point of **3a** was hydrolyzed in acid condition to generate hydroxyl group. ^{1}H NMR spectrum of **4a** [Fig. 2(C)] revealed the complete disappearance of signals assigned to acetal group proton (4.70 ppm) (*a*) and methyl group protons (1.18–1.28 ppm) (*h, i*) after hydrolysis. The successful hydrolysis of acetal group in PEG3 (**3a**) was further confirmed by MALDI-TOF MS analysis (Fig. 3). The spectra for **3a** and **4a** were expanded partly to analyze the hydrolysis reaction. Obviously, the spacing of 44.1 Da between adjacent peaks in each spectrum was equal to the mass of PEG repeating unit. It was observed that a decrease of 72.0 Da from peak 3584.1 Da [PEG3-OCH(CH_3)OC_2H_5·Na^+, cal. 3584.3 Da] to peak 3512.1 Da (PEG3-OH·Na^+, cal. 3512.2 Da) was attributed to the mass of acetal group [—CH(CH_3)OC_2H_5]. The overall MW decrease of 72.0 Da from **3a** to **4a** supported the successful hydrolysis of acetal group. Furthermore, no degradation was found from the spectra analysis.

Synthesis of PEG4 and End Group Modification

The ROP of EO initiated with macroinitiator **4a** and DPMK was also carried out with typical procedure. The solid line in Figure 1 showed the GPC trace of obtained PEG4. Its retention time was less than that of PEG3 trace (dashed line), demonstrating the increase of MW after the second step ROP of EO. Each arm of PEG3 from the first step polymerization showed the same length and was defined as PEG_A, while the fourth arm of PEG4 was defined as PEG_B (Scheme 1). The length ratio of PEG_A and PEG_B can be easily controlled by changing the feeding mass of EO in two steps polymerization to obtain various "umbrella-like" structures. The MW data of three samples prepared by us were listed in Table 1.

PEG4 with hydroxyl group at PEG_B arm end (**5a**) could be converted to PEG4-CN (**6a**) by Michael addition reaction through adding acrylonitrile in alkaline condition, and then to PEG4-$CONH_2$ (**7a**) in strong acid condition, which can be easily hydrolyzed to PEG4-COOH (**8a**) in alkaline environment (pH = 12). In the ^{1}H NMR spectrum for PEG4-CN (**6a**) [Fig. 4(B)], characteristic resonance signal for methylene group adjacent to cyano was observed at δ 2.62 ppm (*m*), which was overlapped with signals of protons of three methylene groups adjacent to nitrogen atom (2.57–2.72 ppm) (*c*, *d*). In the ^{1}H NMR spectrum for PEG4-COOH (**8a**) [Fig. 4(C)], characteristic resonance signal for methylene group adjacent to carboxyl was detected at δ 2.73 ppm (*n*), also overlapped with signals of methylene protons adjacent to nitrogen atom (*c*, *d*). However, the transformation process of hydroxyl end group could be well characterized by MALDI-TOF MS analysis (Fig. 5), indicating the nearly complete conversion of functional groups. In the spectrum for PEG4-OH [Fig. 5(A)], the major series of the molecular masses of PEG4 (**5a**) (peak: ***a***, ***b***, ***c***, ***d***, ***e***) were expressed in the following equation: MW_{MS} = 44.05*n* (EO) + 176.19 ($C_7H_{13}NO_4$) + 273.39 (three benzyl groups) + 22.99 (sodium), whereas the lower ones (peak *e, f, g, h*) were expressed using potassium to substitute sodium in the equation above. The presence of the potassium adduct was due to unsuccessful purification process, because DPMK was used in the polymerization. Moreover, the spacing of 16.1 Da between the nearest peaks was exactly equal to the mass difference of sodium and potassium adducts. In Figure 5, an increase of 53.1 Da from peak *a* (PEG4-OH·Na^+ = 5318.3 Da, cal. 5318.4 Da) to peak *a*′ (PEG4-CN·Na^+ = 5371.4 Da, cal. 5371.5 Da) was ascribed to the mass of acrylonitrile. The overall MW increase of 53.1 Da demonstrated the formation of PEG4-CN (**6a**) through Michael addition reaction. The success of transformation reaction from PEG4-CN (**6a**) to PEG4-COOH (**8a**) with an increase of 18.9 Da from peak *a*′ (PEG4-CN·Na^+ = 5371.4 Da, cal. 5371.5 Da) to peak *a*″ (PEG4-COOH·Na^+ = 5390.3 Da, cal. 5390.4 Da) was also observed.

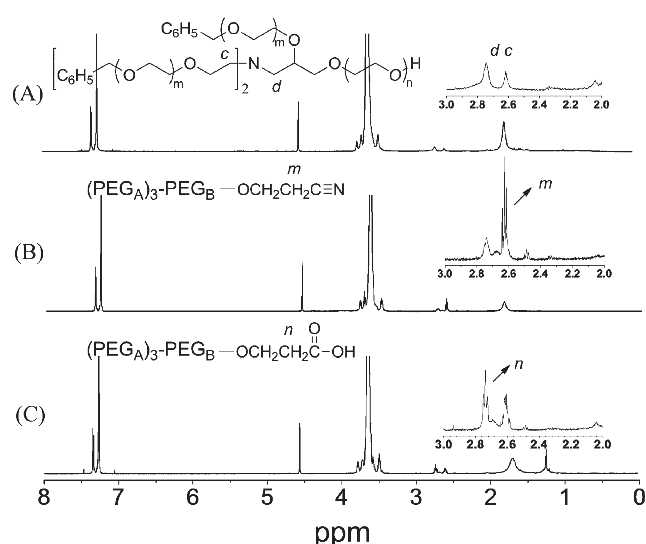

FIGURE 4 ^{1}H NMR spectra of PEG4-OH (A), PEG4-CN (B), and PEG4-COOH (C) (Sample II in Table 1).

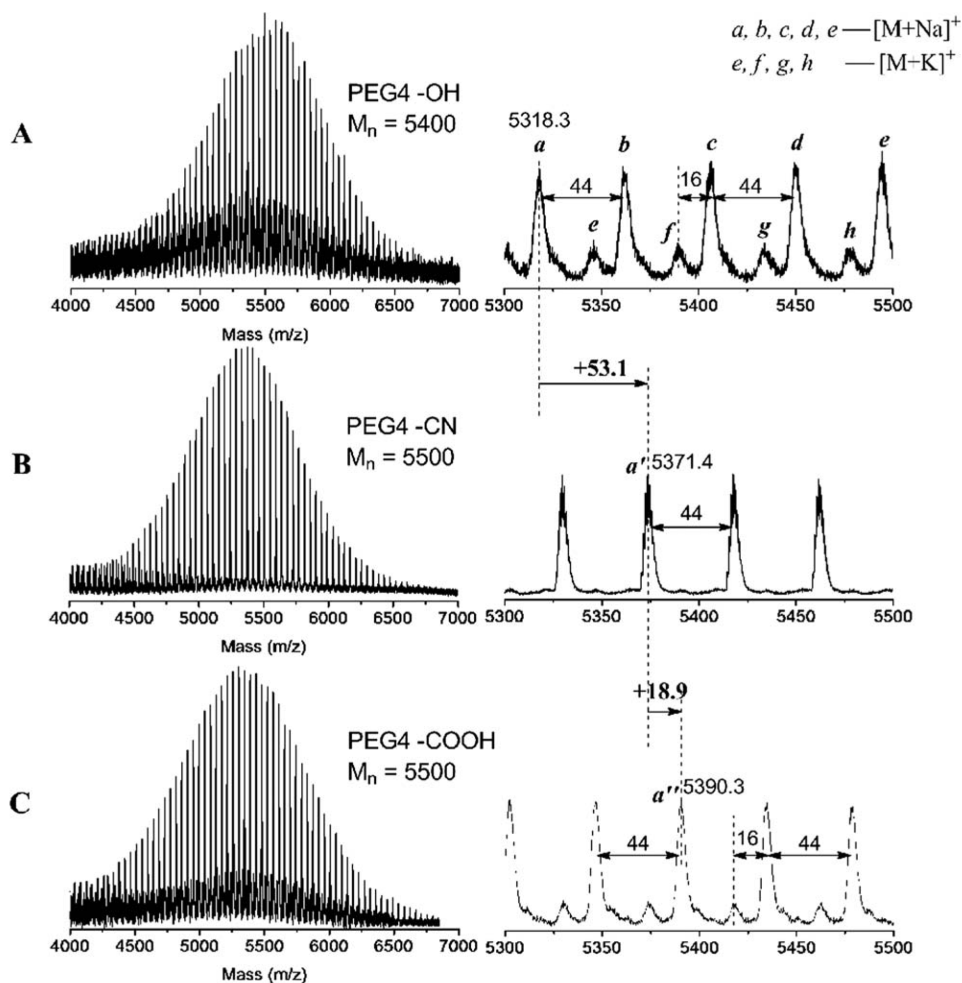

FIGURE 5 MALDI-TOF MS spectra of (A) PEG4-OH (**5a**), (B) PEG4-CN (**6a**), and (C) PEG4-COOH (**8a**).

Bioconjugation of PEG4-SPA to IFN-α-2b

The bioconjugation reaction of PEG4-SPA (10 kDa) and IFN-α-2b was carried out using the standard procedure at pH 9.0 with excess polymer concentration (polymer/protein = 10:1) for 2 h. The PEG4-SPA was attached on surface exposed ε-amino group of lysine residues via an amide bond. After purification using ion-exchange chromatography (IEC) and Macro-Prep High S Cartridges 1 mL (Bio-Rad), mono-PEG4-IFN-α-2b was collected and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 6) and high-performance liquid chromatography (HPLC) (Fig. 7). SDS-PAGE analysis demonstrated a clear shift of the conjugates to higher molecular weight (lane 3, 4) when compared to native IFN-α-2b (lane 2). The PEGylation products consisted of mono-PEG4-IFN-α-2b, multi-PEG4-IFN-α-2b (conjugates with multiple polymers attached) as expected based on the plurality of available amino residues, as well as unconjugated IFN-α-2b and unreacted PEG4. The only one species for the purified products with high molecular weight was observed in lane 4 after separation. In Figure 7, the solid line and dashed line illustrated purified PEG4-IFN-α-2b and IFN-α-2b, respectively. The single peak of purified PEG4-IFN-α-2b also indicated the successful purifi-

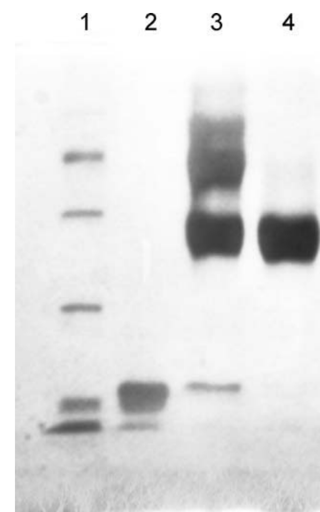

FIGURE 6 SDS-PAGE results from the reactions of PEG4-SPA with IFN-α-2b in phosphate buffer at pH 9.0. Lanes: 1, molecular weight marker proteins; 2, native IFN-α-2b; 3, PEGylation reaction mixture; 4, purified PEG4-IFN-α-2b.

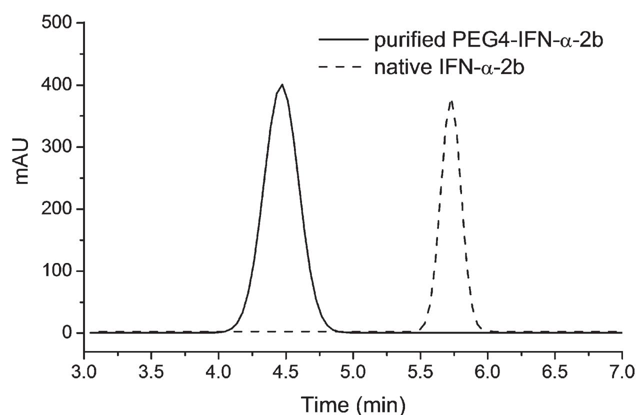

FIGURE 7 HPLC of purified PEG4-IFN-α-2b using IEC and IFN-α-2b.

cation of the PEGylation mixture without existence of impurities.

EXPERIMENTAL

Materials

EO, Sinopharm Chemical Reagent (SCR), China, was dried over CaH_2 for 48 h and then distilled under N_2, stored at −20 °C before use. Diethanolamine (99%, SCR), ethyl bromide (98%, Aldrich), benzyl bromide (98%, Aldrich), NHS (97%, SCR), and dicyclohexylcarbodiimide (DCC, 95%, Sheshan chemical factory, China) were used as received. Other reagents were purchased from SCR and purified by conventional methods before use. Silica gel plates (HSGF254, Jiangyou Silica Gel Development, Yantai, China) were used for thin-layer chromatographic (TLC) analysis. EEGE (**1**) was synthesized from glycidol and ethyl vinyl ether according to Fitton et al.[33] and distilled under reduced pressure (b.p. 152–154 °C) with the purity of >99.6% (GC).[35] Diphenylmethyl potassium (DPMK) solution was freshly prepared by the reaction of potassium naphthalenide with diphenylmethane in THF according the literature,[36] and the concentration was 0.52 M. IFN-α-2b was supplied by Harbin Pharmaceutical Group, China.

Instrumentation

GPC was performed in 0.1 M aqueous $NaNO_3$ at 40 °C with an elution rate of 0.5 mL/min on an Agilent 1100 with a G1310A pump, a G1362A refractive index detector and a G1315A diode-array detector calibrated by PEO as standard samples. ^{1}H NMR spectra were obtained on a DMX 500 MHz spectrometer with tetramethylsilane as the internal standard and $CDCl_3$ as the solvent, except for bHAEPO (**2**) which was measured in CD_3OD. The matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) measurement was performed using a Perspective Biosystem Voyager-DE STR MALDI-TOF MS (PE Applied Biosystems, Framingham, MA). Matrix solution of dithranol (20 mg/mL), polymer (10 mg/mL), and cationizing salt of sodium trifluoroacetate (10 mg/mL) in THF were mixed in the ratio of matrix: cationizing salt: polymer = 10:1:2 and 0.8 μL of mixed solution was deposited on the sample holder. All mass spectra were recorded in the reflector mode. UV spectra were taken on a 756 MC ultraviolet–visible light spectrophotometer (Shanghai Third Analytical Instrument Factory, China). IEC was carried out using SOURCE 15Q (GE Healthcare, Shanghai) packed column and Macro-Prep High S Cartridges 1 mL (Bio-Rad) connected to HPLC system (1100 Series, Agilent). SDS-PAGE was carried out with 4–15% Tris-HCl gels (Bio-Rad, 1.0 mm × 10 well).

Synthesis of bHAEPO (2)

A solution of diethanolamine (11.0 g, 0.105 mol) in 15 mL anhydrous methanol was added dropwise into the solution of EEGE (**1**) (18.0 g, 0.123 mol) and THF (45 mL) under nitrogen protection at 0 °C with vigorous stirring. The mixture was stirred for another 60 h at 10 °C. TLC analysis [ethyl ether/cyclohexane (v/v = 1:1) as mobile phase] was used to trace whether the reaction was complete. After the solvents were removed under reduced pressure, the residue was dissolved in 200-mL deionized water and purified by extraction with ethyl ether (100 mL × 2) to remove the unreacted EEGE. The aqueous layer was concentrated under reduced pressure, then the remainder was dissolved in 100-mL THF and dried with $MgSO_4$ for 24 h. After filtration, the solvent was evaporated and the product was obtained as colorless oil.

Yield: 24.5 g, 84%. ^{1}H NMR (CD_3OD), δ (ppm): 1.18 (t, C***H*₃**CH₂O—), 1.28 (d, C***H*₃**—CH—), 2.57–2.72 (m, —C***H*₂**—N—(C***H*₂**)₂—), 3.56–3.66 (m, —C***H*₂**—), 3.82 (m, —C***H***—OH), 4.71 (q, —OC***H***(CH₃)O—). Elem. Anal. ($C_{11}H_{25}NO_5$): required C 52.57, H 10.03, N 5.57; found C 49.69, H 10.03, N 5.30.

Synthesis of Terminated PEG3 (3a and 3b)

The polymerization was carried out in a stainless steel kettle and the typical procedure was described as follows: a 400-mL kettle was vacuumed at 80 °C for 24 h and cooled to room temperature and then to −20 °C. A given volume of an initiator solution [bHAEPO (**2**) (5.1 g, 20.30 mmol) with DPMK (35.1 mL, 18.27 mmol) dissolved in DMSO (120 mL)] and EO (63.0 g, 1.43 mol) was introduced successively into the kettle. Subsequently, it was heated to 50 °C under stirring for 48 h. After the reaction was completed, an additional DPMK solution (82.0 mL, 42.63 mmol) was injected into the system to guarantee the complete deprotonation of all hydroxyl groups, then terminated with excess benzyl bromide (14.5 mL, 0.122 mol). The obtained solution was stirred at 50 °C for another 24 h and the solvent was evaporated, subsequently, the salt was removed by filtration and the benzyl-terminated PEG3 (**3a**) were was obtained by precipitation in cold ethyl ether twice and dried under vacuum at 45 °C.

Yield: 32.8 g (87%). GPC: $M_{n(GPC)}$ = 3000 Da, polydispersity index (PDI) = 1.09. ^{1}H NMR ($CDCl_3$), δ (ppm): 1.18 (t, C***H*₃**-CH₂O—), 1.28 (d, C***H*₃**—CH—), 2.61–2.75 (m, —C***H*₂**—N—(C***H*₂**)₂—), 3.51–3.77 (m, 4H, —C***H*₂**C***H*₂**O— of PEG backbone), 4.58 (s, —C***H*₂**—C₆H₅), 4.70 (q, —OC***H***(CH₃)O—), 7.33 (m, —CH₂—C₆***H*₅**). MALDI-TOF MS: $M_{n(MALDI\text{-}TOF\ MS)}$ = 3580 Da.

The ethyl-terminated PEG3 (**3b**) was obtained by the similar procedure using ethyl bromide to substitute benzyl bromide (see Supporting Information).

Yield: 32.0 g (85%). GPC: $M_{n(GPC)} = 3000$ Da, PDI = 1.08. ^{1}H NMR ($CDCl_3$), δ (ppm): 1.18 (m, $C\boldsymbol{H_3}CH_2O$—), 1.28 (d, $C\boldsymbol{H_3}$—CH—), 2.61–2.75 (m, —$C\boldsymbol{H_2}$—N—$(C\boldsymbol{H_2})_2$—), 3.50–3.77 (m, 4H, —$C\boldsymbol{H_2}C\boldsymbol{H_2}O$— of PEG backbone and $CH_3C\boldsymbol{H_2}O$—), 4.70 (q, —$OC\boldsymbol{H}(CH_3)O$—).

Hydrolysis for Synthesis of PEG3-OH (4a)

A mixture of **3a** (10.0 g, 2.8 mmol, $M_{n(MALDI\text{-}TOF\ MS)} = 3580$ Da), methanol (15 mL) and concentrated hydrochloric acid (15 mL) was stirred at room temperature for 2 h. The pH of the solution was adjusted to 3.0 by addition of potassium hydroxide solution (5.0 M). The product was extracted with CH_2Cl_2 (200 mL × 2), then the organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The product **4a** was obtained by precipitation in cold ethyl ether twice and dried under vacuum at 45 °C.

Yield: 8.7 g (89%). ^{1}H NMR ($CDCl_3$), δ (ppm): 2.61–2.75 (m, —$C\boldsymbol{H_2}$—N—$(C\boldsymbol{H_2})_2$—), 3.51–3.77 (m, 4H, —$C\boldsymbol{H_2}C\boldsymbol{H_2}O$— of PEG backbone), 4.58 (s, —$C\boldsymbol{H_2}$—C_6H_5), 7.33 (m, —$CH_2C_6\boldsymbol{H_5}$). MALDI-TOF MS: $M_{n(MALDI\text{-}TOF\ MS)} = 3500$ Da.

Synthesis of PEG4 (5a)

The polymerization procedure was similar to the synthesis of PEG3. A given volume of an initiator solution [**4a** (6.0 g, 1.71 mmol, $M_{n(MALDI\text{-}TOF\ MS)} = 3500$ Da) with DPMK solution (1.0 mL, 0.51 mmol) dissolved in DMSO (60 mL)] and EO (4.0 g, 91 mmol) were introduced successively into a dried 300 mL ampoule. Subsequently, it was heated to 50 °C under stirring for 48 h. After termination with methanol and precipitation in cold ethyl ether twice, the yellowish product, PEG4 (**5a**) was obtained and dried under vacuum at 45 °C.

Yield: 8.7 g, 87%. GPC: $M_{n(GPC)} = 4900$ Da, PDI = 1.11. ^{1}H NMR ($CDCl_3$), δ (ppm): 2.61–2.75 (m, —$C\boldsymbol{H_2}$—N—$(C\boldsymbol{H_2})_2$—), 3.51–3.77 (m, 4H, —$C\boldsymbol{H_2}C\boldsymbol{H_2}O$— of PEG backbone), 4.58 (s, —$C\boldsymbol{H_2}$—C_6H_5), 7.33 (m, —$CH_2C_6\boldsymbol{H_5}$); MALDI-TOF MS: $M_{n(MALDI\text{-}TOF\ MS)} = 5400$ Da.

Synthesis of PEG4-CN (6a)

A mixture of **5a** (5.0 g, 0.93 mmol, $M_{n(MALDI\text{-}TOF\ MS)} = 5400$ Da), anhydrous 1,4-dioxane (30 mL) and potassium hydroxide (0.6 g) was cooled to 0–5 °C in an ice bath. Acrylonitrile (0.5 g, 9.30 mmol) was added dropwise within 0.5 h, and the solution was stirred for 3 h at 0–5 °C. The pH of the solution was adjusted to 7.0 by addition of sodium phosphate. The products were extracted with CH_2Cl_2 (100 mL × 2), and then the organic layer was dried over $MgSO_4$. Thus, PEG4-CN (**6a**) was obtained by precipitation in cold ethyl ether twice and dried under vacuum at 45 °C.

Yield: 4.5 g, 90%. ^{1}H NMR ($CDCl_3$), δ (ppm): 2.61–2.75 (m, —$C\boldsymbol{H_2}$—N—$(C\boldsymbol{H_2})_2$—), 2.62 (t, —$C\boldsymbol{H_2}$—CN), 3.51–3.77 (m, —$C\boldsymbol{H_2}C\boldsymbol{H_2}O$— of PEG backbone), 4.58 (s, —$C\boldsymbol{H_2}$—C_6H_5), 7.33 (m, —$CH_2C_6\boldsymbol{H_5}$); MOLDI-TOF MS: $M_{n(MALDI\text{-}TOF\ MS)} = 5500$ Da.

Synthesis of PEG4-CONH$_2$ (7a) and PEG4-COOH (8a)

A mixture of **6a** (4.0 g, 0.73 mmol, $M_{n(MALDI\text{-}TOF\ MS)} = 5500$ g/mol) and concentrated hydrochloric acid (25 mL) was stirred for 48 h at 25 °C. PEG4-CONH$_2$ (**7a**) was not separated and was converted to PEG4-COOH (**8a**) directly as described as follows. The solution was diluted with 200 mL water and potassium hydroxide (20.0 g) was added, and the mixture was stirred for 22 h at 25 °C. After neutralization with 1.0 M hydrochloric acid (~60 mL), the solution was extracted with CH_2Cl_2 (100 mL × 3). The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated by rotary evaporation. The products were collected by precipitation in cold ethyl ether twice and dried under vacuum at 45 °C.

Yield: 3.2 g, 80%. ^{1}H NMR ($CDCl_3$), δ (ppm): 2.61–2.75 (m, —$C\boldsymbol{H_2}$—N—$(C\boldsymbol{H_2})_2$—), 2.62 (t, —$C\boldsymbol{H_2}$—COOH), 3.51–3.77 (m, —$C\boldsymbol{H_2}C\boldsymbol{H_2}O$— of PEG backbone), 4.58 (s, —$C\boldsymbol{H_2}$—C_6H_5), 7.33 (m, —$CH_2C_6\boldsymbol{H_5}$); MOLDI-TOF MS: $M_{n(MALDI\text{-}TOF\ MS)} = 5500$ Da.

Synthesis of PEG4-SPA (9a)

7a (3.0 g, 0.55 mmol, $M_{n(MALDI\text{-}TOF\ MS)} = 5500$ Da) was dissolved in CH_2Cl_2 (15 mL), and NHS (0.127 g, 1.10 mmol) was added. The solution was cooled to 0 °C. A solution of DCC (0.227 g, 1.10 mmol) in CH_2Cl_2 (5 mL) was added dropwise, and the solution was stirred at 25 °C overnight. The reaction mixture was filtered, concentrated and precipitated by addition to cold ethyl ether twice.

Yield: 2.6 g, 86.7%. The NHS content of PEG-SPA was calculated by NHS absorption at 260 nm of UV spectra, which was carried out by hydrolysis of PEG-SPA.[37] The value of content was 98%.

Preparation and Purification of PEG4-IFN-α-2b Conjugates (10a)

PEGylation reaction was conducted in 0.1-M phosphate-buffered saline, pH 9.0, with IFN-α-2b (5–8 mg/mL) and PEG4-SPA (10 kDa) reagent at 1:10 molar ratio. Reaction mixture was stirred for 2 h at 4 °C, and the reaction was stopped by adjusting the pH of the mixture to 4.5 with glacial acetic acid. The polymer–protein conjugates were purified by IEC. The reaction mixture was applied onto a column packed with SOURCE 15Q, operated on HPLC system, previously equilibrated with 20-mM Tris-HCl, pH 8.5, using a gradient of increasing sodium chloride in equilibration buffer, pH 8.5, at flow rate of 3.0 mL/min. Eluting the matrix gave several fractions, and each fraction was analyzed by SDS-PAGE according to the literatures.[38,39] Then, the PEG4-IFN-α-2b fraction was further purified by Bio-Scale High S Cartridge connected to HPLC system, previously equilibrated with at least 10-column volumes of 20 mM NaAc-HAc/2.0 M NaCl, pH 4.4, using a gradient of decreasing sodium chloride in equilibration buffer, pH 4.4. The main fraction was monoPEG4-IFN-α-2b conjugates with high purity by SDS-PAGE analysis. The modification degree and the purity of the PEG4-IFN-α-2b were determined by size exclusion HPLC (GF-250 4.6 × 250 mm).

CONCLUSIONS

In summary, monofunctional "umbrella-like" PEG derivatives were successfully synthesized via two-step anion ROP of EO using binary initiation system of bHAEPO and DPMK. The well-defined structures were characterized by GPC, ^{1}H NMR, and MALDI-TOF MS analysis, demonstrating the feasibility of

this straightforward synthetic route. Subsequent conjugation with protein (IFN-α-2b) showed successful coupling reaction between the bioreactive group of PEG4 and the amino group of lysine residues on the IFN-α-2b surface.

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