Useful Synthetic Method of Polypeptides with Well-Defined Structure by Polymerization of Activated Urethane Derivatives of α-Amino Acids

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INTRODUCTION Polypeptides have received great attention as promising components for new design of various macromolecular architectures,[1–5] because of its intriguing feature such as α-helix and β-sheet. For polypeptide synthesis, various synthetic strategies have been developed. Small peptide sequences were generally synthesized by stepwise solid-phase synthesis, whereas, for synthesis of high molecular weight polypeptides, living ring-opening polymerizations of *N*-carboxyanhydride (NCA) of α-amino acid are widely used. The polymerization of NCA was usually initiated by nucleophiles such as primary or secondly amines, which lead to the production of polypeptides with well-defined chain length and terminal structure. Therefore, its polymerization system could allow to construct various kinds of polypeptides-based functional materials, including drug delivery,[6–8] tissue engineering,[9] surface modification,[10–12] and organic electronics.[13,14]

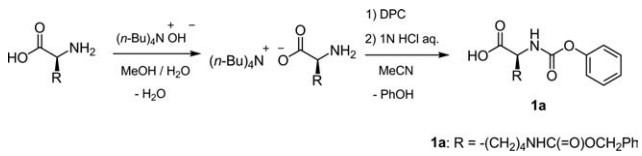
Although living polymerization of NCA affords an important advantage in synthesis of well-defined polypeptide, the susceptive nature of NCA for moisture and heating is major drawbacks for its production toward practical use. Thus, development of alternative procedure for polypeptide synthesis is a great challenge in this research area. To date, some useful monomers for polypeptides synthesis have been reported. For example, Hurd and Buess developed α-carboxy hydroxamic acid for synthesis of polypeptides,[15] and Ehler and Orgel used *N*-imidazolylcarboxyl amino acid as precursor of NCA.[16] Also, we previously reported selective synthesis of NCA and polypeptides through *N*-(4-nitrophenyloxycarbonyl) amino acid.[17–22]

Recently, we have developed newly synthetic route for production of NCA through intramolecular cyclization of *N*-(phenyloxycarbonyl) amino acids under heating in moderately polar solvents, such as 2-butanone, tetrahydrofuran (THF), and acetonitrile to give the corresponding NCA, without the use and production of any toxic compounds.[23] In this study, we report the polypeptides synthesis with well-defined terminal structure, using *N*-(phenyloxycarbonyl) amino acid as precursor of NCA in the presence of amine, and approaching for the synthesis of block copolypeptides. In this synthetic method, these urethane derivatives were easily synthesized with *N*-carbamoylation of α-amino acid with diphenyl carbonate (DPC) and stable for moisture or heating. Therefore, polypeptides synthesis using urethane derivatives is more convenient than conventional ring opening polymerization of highly reactive NCAs, for application to various functional materials.

RESULTS AND DISCUSSION

The general synthetic routes toward urethane derivatives of α-amino acids are outlined in Scheme 1. According to previous our report, we synthesized *N*-(phenyloxycarbonyl) amino acids by two step reactions: (1) transformation of α-amino acids into the corresponding ammonium salts and (2) *N*-carbamoylation of the salts with DPC. After carefully purification by column chromatography and recrystallization, urethane derivatives of α-amino acid were successfully obtained in moderate yield (68%). These urethane derivatives of α-amino acid could be stored stably for several months at room temperature.

To investigate polymerization behavior of their urethane derivatives in details, as illustrated in Scheme 2, polymerization of **1a** was first examined in *N*,*N*-dimethylacetamide (DMAc) at 60 °C for 48 h without any additives. The time-conversion relationship of **1a** was tracked with ^{1}H NMR analysis, by which completely consumption of **1a** within 48 h (Fig. 1) and production of the corresponding poly-Z-L-lysine (PZLL). During polymerization process, ^{1}H NMR spectra clearly detected a small amount of Z-L-lysine-*N*-carboxyanhydride (ZLL-NCA). The obtained polypeptides (**2a**) were collected as ether-insoluble parts in moderate yield (65%). The size exclusion chromatography (SEC) analysis revealed that isolated polypeptides had number-average molecular weight (M_n) of 56,000 and wide polydispersity index (M_w/M_n =

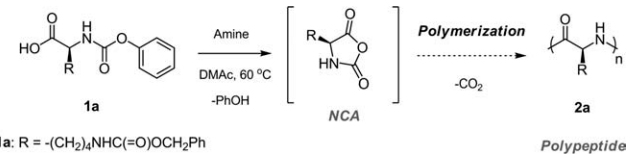


SCHEME 1 Synthetic routes for urethane derivatives of α-amino acids (**1a**).

2.83). These results might indicate that the polypeptides were synthesized through various reaction pathways. Second, polymerizations in the presence of *n*-butylamine (*n*-$BuNH_2$) (feed ratio $[1]_0/[n\text{-}BuNH_2]_0 = 50$) were investigated in DMAc at 60 °C (entry 2, in Table 1). From ^1H NMR analysis of **1a** in reaction mixture, it was observed that consumption of **1a** was dramatically accelerated in the presence of *n*-$BuNH_2$ (Fig. 1). The corresponding polypeptides were obtained as ether-insoluble parts in excellent yield (89%). In the SEC analysis, the obtained polypeptides possessed M_n of 15,600 and narrow M_w/M_n of 1.28. Furthermore, the polymerization of **1a** was carried out with various feed ratio of *n*-$BuNH_2$, and their results were summarized in Table 1. Intriguingly, the M_n of obtained polypeptides could be controlled well as varying the initial ratio of **1a** to *n*-$BuNH_2$, in the range of 8000–28,000, and each polypeptides showed narrow molecular weight distribution ($M_w/M_n = 1.28$–1.34). Also, the acceleration effects could be highly observed as the amount of *n*-$BuNH_2$ increased for **1a** (Fig. 1).

To determine terminal-structure of polypeptides, MALDI-TOF-mass spectroscopy analysis was carried out, using 2,5-dihydroxybenzoic acid and sodium trifluoroacetate, as a matrix material and a cationization agent, respectively. In the spectrum of polypeptides synthesized with *n*-$BuNH_2$, significant signals were observed at *m/z* value of 1145, 1408, 1670, 1932, 2194, 2456, and 2718, as Na^+ adducts (Fig. 2). These signals were regularly located with an interval of 262, which corresponds to the formula weight of ZLL. From these results of MALDI-TOF-mass spectra, we could presumed that the resulting polypeptides possessed *n*-$BuNH_2$-incorporated initiating end and —NH_2 group at propagating end without any side reactions terminating chain growth. These results suggested that sterically unhindered *n*-$BuNH_2$ was successfully reacted with formed NCA in the initiating stage, resulting in production of polypeptides with well-defined terminal structure.

In addition, property [optical rotation and circular dichroism (CD) spectra] of synthesized PZLL was compared with authentic sample, which was synthesized by ring opening polymerization of ZLL-NCA, to make it clear that no racemization is taking place under reaction condition. Optical rotation



SCHEME 2 Polypeptides synthesis using urethane derivatives of α-amino acids (**1a**) in the presence of amines.

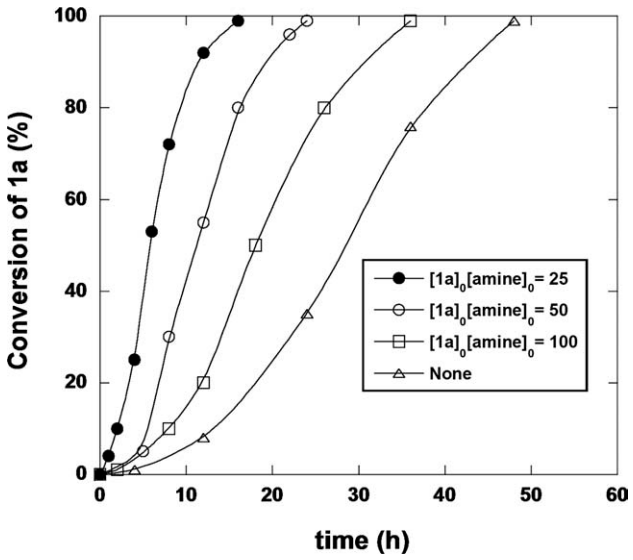


FIGURE 1 Time-conversion relationship of **1a** in DMAc, at 60 °C in the absence or presence of *n*-$BuNH_2$.

of the PZLL (entry 3, in Table 1) and the authentic sample (M_n: 12,500, $M_w/M_n = 1.18$) was $[\alpha]^{22}_D$: 3.8 ($c = 0.1$, $CHCl_3$) and $[\alpha]^{22}_D$: 4.2 ($c = 0.1$, $CHCl_3$), respectively. The lysine side chains were deprotected by stirring under acidic condition to determine optical rotation, and second, structure of poly-L-lysine (PLL) in aqueous solution. Each deprotected sample (PLL prepared from urethane derivatives or ring opening polymerization of ZLL-NCA) also showed close values, $[\alpha]^{22}_D$: −65.0 ($c = 0.1$, H_2O) and $[\alpha]^{22}_D$: −66.8 ($c = 0.1$, H_2O), respectively. The CD spectra of PZLL and PLL were obtained in CH_2Cl_2 and aqueous solution (Fig. 3). In CH_2Cl_2 solutions, CD spectra of each sample showed a negative cotton effect with similar intensity, suggesting the formation of α-helical polypeptides, while positive band at around 220 nm and negative one at 196 nm, which is attributed to random coil conformation, were observed in aqueous solution of polypeptides. From the results of optical rotation and CD spectra of polypeptides, it was confirmed that the present polymerization system affords well-defined polypeptides without any racemization of chiral centers.

TABLE 1 Polymerization of **1a** in DMAc, at 60 °C in the Presence of *n*-$BuNH_2$[a]

Entry	Feed Ratio $[\mathbf{1a}]_0/[\text{amine}]_0$	Conv. (%)[b]	Yield (%)[c]	M_n[d]	M_w/M_n[d]
1	None	>99	65	56,000	2.83
2	25	>99	89	8,700	1.30
3	50	>99	87	15,600	1.28
4	100	>99	84	28,000	1.34

[a] Polymerization condition: $[\mathbf{1a}]_0 = 1.0$M.
[b] Calculated by ^1H NMR spectra.
[c] Ether-insoluble parts.
[d] Estimated by SEC [eluent: DMF solution of LiBr (10 mM), calibrated by polystyrene standards].

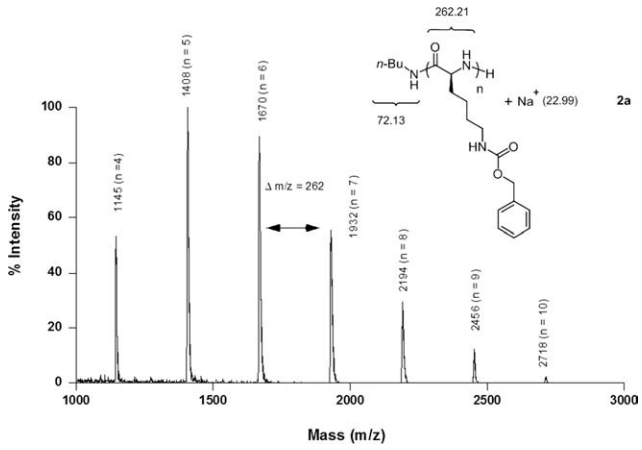
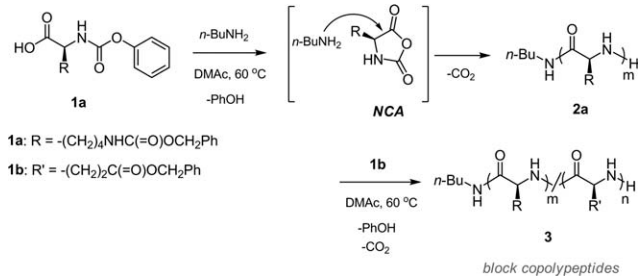


FIGURE 2 MALDI-TOF-mass spectrum of PZLL obtained by polymerization of **1a** in the presence of *n*-$BuNH_2$ ($[\mathbf{1a}]_0/[amine]_0 = 50$).



SCHEME 3 Synthesis of block copolypeptides using urethane derivatives of α-amino acids in the presence of *n*-$BuNH_2$.

Furthermore, synthesis of block copolymer with α-amino acid components, using the present polymerization system, was carried out (Scheme 3). The polypeptide of ZLL was first synthesized from urethane derivatives **1a** in the presence of *n*-$BuNH_2$ in DMAc at 60 °C. After complete consumption of **1a**, other urethane derivatives such as γ-benzyl-L-glutamate (BLG) **1b** were added into the reaction mixture to obtain the corresponding block copolypeptides (**3**). As expected, obvious chain growth from terminal group of PZLL was observed in SEC analysis (Fig. 4), whereas molecular weight distribution remained relatively narrow values (M_w/M_n = 1.40–1.55) (Table 2). ^{1}H HMR spectra of the block copolypepitdes exhibited newly significant peaks, which is attributed to BLG moieties. The composition ratio of block copolypeptides was easily adjusted by varying additional amount of urethane derivatives. Block copolymerization with BLG moieties gave soluble polypeptides in the common organic solvents such as, *N*,*N*-dimethylformamide (DMF), THF, acetone, and chloroform. This present polymerization system can be extended to more facile synthesis of various polypeptides with well-defined structures, compared with conventional ring opening polymerization of NCA.

SUMMARY

We reported facile polymerization strategy for synthesis of polypeptides with well-defined structure using urethane derivatives of α-amino acids in the presence of *n*-$BuNH_2$. The molecular weight of polypeptides was adjusted by varying the initial ratio of **1a** to *n*-$BuNH_2$, and molecular weight distribution remained narrow values (below 1.35). The MALDI-TOF-mass spectroscopy revealed that the obtained polypeptides had *n*-$BuNH_2$-incorporated initiating end and —NH_2 group at propagating end without any side reactions terminating chain growth. The synthetic procedure for block copolypeptides (**3**) was established by polymerization of **1a** in the presence of *n*-$BuNH_2$, followed by subsequent addition of other urethane derivatives **1b**. This polymerization system newly provides more facile synthesis of polypeptides with well-defined structure for application to important

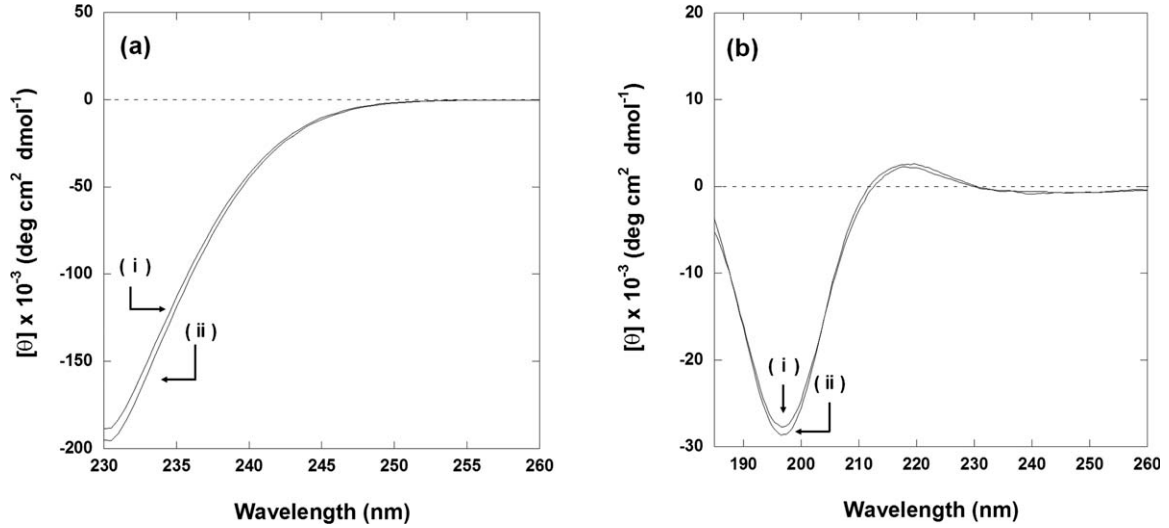


FIGURE 3 CD spectra of PZLL (a) in CH_2Cl_2 (0.5 mg/mL): (i) PZLL prepared from urethane derivatives (entry 3, in Table 1), (ii) PZLL prepared by ring-opening polymerization of ZLL-NCA, and PLL (b) in H_2O (0.25 mg/mL): (i) PLL derived from urethane derivatives and subsequent deprotection, (ii) PLL derived from ring-opening polymerization of ZLL-NCA and subsequent deprotection.

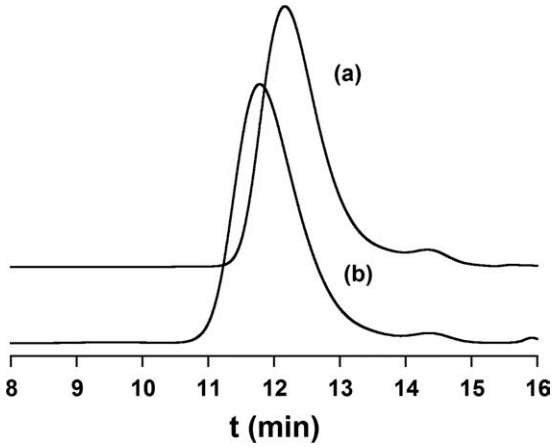


FIGURE 4 SEC results of PZLL (a) and PZLL-*b*-PBLG (b), obtained by urethane derivatives of **1a** and **1b** (entry 1, in Table 2).

biomaterials, compared with conventional ring opening polymerization of NCA.

EXPERIMENTAL

Generals

All reagents used were purchased from Aldrich and Tokyo Chemical Industry and used as received without further purification. Butylamine (*n*-$BuNH_2$) and DMAc were purified by heating at 60 °C for 1 h under calcium hydride (CaH_2), followed by fractional distillation before use. *N*-(phenoxycarbonyl)-BLG (**1b**) was synthesized by the reaction of BLG with phenyl chloroformate, according to the procedures previously reported.[20] ^1H and ^{13}C NMR spectra were recorded with a JEOL ECS-400 (400 MHz) spectrometer, and chemical shifts were recorded in ppm units using tetremethylsilane as an internal standard. IR spectra were taken with KBr disks on a Nicolet iS10 FT-IR, Thermo Scientific, Japan. Elemental analysis was performed with a LECO CHNS-932 analyzer. Optical rotation was measured with a JASCO DIP-1000 digital polarimeter. For the determination of number-average molecular weight (M_n) and polydispersity index (M_w/M_n) for synthesized polypeptides, SEC was performed 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 °C. The system was operated using 10 mM LiBr in DMF as the eluent, at a flow rate of 0.5 mL/min. Polystyrene standards were used for calibration. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was carried out on a PerSeptive Biosystems Voyager DE Pro Bio Spectrometry workstation. The samples were prepared using 2,5-dihydroxybenzoic acid and sodium trifluoroacetate, as a matrix material and a cationization agent, respectively. The resulting samples were irradiated with 337 nm of nitrogen laser, and detected on positive mode at 20 kV. CD spectroscopy was used to determine the secondary structure of synthesized polypeptides on a JASCO J-720WI spectropolarimeter. Melting point was measured on Bibby Stuart scientific melting point apparatus SMP3.

Synthesis of Urethane Derivatives of N_ε-Carbobenzoxy-L-lysine (1a)

To a stirred solution of N_ε-carbobenzoxy-L-lysine (ZLL) (3.5 g, 12.5 mmol) in methanol (35 mL) and distilled water (5 mL), tetrabutylammonium hydroxide (37% in methanol) (8.77 g, 12.5 mmol) was slowly added at room temperature. After stirring for 1 h, the reaction mixture was concentrated under reduced pressure. The resulting residues were dissolved in acetonitrile (25 mL). The solution was added dropwise over 10 min to a stirred solution of DPC (2.68 g, 12.5 mmol) in acetonitrile (25 mL) at ambient condition, and then the reaction mixture was stirred overnight. To the reaction mixture, 1N of HCl aqueous solution (20 mL) was added. The mixture was transferred into a separatory funnel containing distilled water (30 mL) and ethyl acetate (30 mL). The aqueous layer was extracted with ethyl acetate (3×30 mL) and the organic fractions were combined, dried over Na_2SO_4, filtrated, and concentrated under reduced pressure. The crude products were purified by flash column chromatography (chloroform:acetone:acetic acid = 7:3:0.1, as eluent), and then recrystallization with ethyl acetate/*n*-hexane to yield 3.4 g (68%) of **1a**, as white solids, mp: 119.8–120.2 °C. $[\alpha]^{22}_D$: 25.8 ($c = 0.1$, $CHCl_3$).

^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 1.34–1.58 (m, 4H), 1.67–1.83 (m, 1H), 1.84–1.96 (m, 1H), 3.15 (m, 2H), 4.39 (m, 1H), 4.86(s, 1H), 5.08 (s, 2H), 5.84 (d, 1H, $J = 8.1$ Hz),, 7.09 (d, 2H, $J = 7.8$ Hz), 7.15 (t, 1H, $J = 7.4$ Hz), 7.28–7.37 (m, 7H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 22.17, 29.33, 31.57, 40.41, 53.78, 66.87, 121.53, 125.42, 128.07, 128.09, 128.48, 129.23, 136.30, 150.72, 154.67, 156.87, 175.82. IR (KBr, thin

TABLE 2 Synthesis of Block Copolypeptides Using Urethane Derivatives 1a and 1b

Entry	Feed Ratio $[\mathbf{1a}]_0/[\text{amine}]_0$	Homopolypeptide $M_n(M_w/M_n)^a$	Feed Ratio $[\mathbf{1b}]_0/[\text{amine}]$	Block Copolypeptide $M_n(M_w/M_n)^a$	Conv. (%)[b]	Yield (%)[c]
1	50	16,000 (1.29)	25	23,000 (1.40)	>>9/>99	86
2	100	26,000 (1.32)	40	31,000 (1.48)	>99/>99	78
3	25	8,000 (1.30)	25	13,500 (1.55)	>99/>99	84

[a] Estimated by SEC [eluent: DMF solution of LiBr (10 mM), calibrated by polystyrene standards].

[b] Calculated by ^1H NMR spectra.

[c] Ether-insoluble parts.

film, cm^{-1}): 3385, 3332, 3063, 2942, 1726, 1689, 1534, 1492, 1407, 1266, 1218, 1183, 1145. Anal. Calcd. for $C_{21}H_{24}N_2O_6$: C, 62.99; H, 6.04; N, 7.00. Found: C, 62.65; H, 5.82; N, 7.00.

Synthesis of Polypeptides by Polymerization of Urethane Derivatives in the Presence of Amines (2a)

Typical procedure is as follows: Urethane derivatives of ZLL (**1a**) (0.4 g, 1.0 mmol) were dissolved in DMAc (1 mL), and then added into flame-dried Schlenk tube, followed by addition of *n*-$BuNH_2$/DMAc solution (20 μL, 1.0 $\times$ 10^{-3} mmol/μL). The polymerization was performed at 60 °C for 48 h under argon atmosphere. After the reaction mixture was cooled to room temperature, it was poured into diethyl ether. The resulting precipitates were collected by filtration, and then dried under vacuum to yield 228 mg (87%) of PZLL (**2a**), as white solids.

^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 1.29–2.09 (br, 6H), 3.15 (s, 2H), 3.85 (s, 1H), 4.98 (s, 2H), 5.49 (s, 1H), 7.16–7.35 (br, 5H).

Synthesis of Block Copolypeptides by Polymerization of Urethane Derivatives (3)

The general polymerization procedures were followed as described above. Urethane derivatives of ZLL (**1a**) (0.4 g, 1.0 mmol) were polymerized in DMAc (1 mL) with *n*-$BuNH_2$ (0.02 mmol) at 60 °C for 48 h under argon atmosphere. An aliquot of reaction mixtures were withdrawn for SEC analysis. After complete consumption of urethane derivatives, DMAc solution of **1b** (1 mL, 0.5 mol/L, 0.5 mmol) was added to the reaction mixture containing PZLL (**2a**) for second block. The resulting block copolypeptides were reprecipitated by diethyl ether, followed by dried under vacuum to yield 414 mg (86%) of PZLL-*b*-PBLG (**3**) as white solids.

Synthesis of Z-L-Lysine-*N*-Carboxyanhydride (ZLL-NCA)

According to previous our reports,[18,23] urethane derivatives of ZLL (**1a**) (1.6 g, 4 mmol) and acetic acid (0.72 g, 12 mmol) were dissolved in dry 2-butanone (40 mL) and refluxed for 24 h under argon atmosphere. After the reaction mixture was cooled to room temperature, the reaction mixture was concentrated. The crude products were purified by flash column chromatography under dry condition (eluting with a gradient from 50% to 70% ethyl acetate in *n*-hexane) and then recrystallization with THF/*n*-hexane to yield 610 mg (50%) of ZLL-NCA, as white powder, mp: 98.2–99.4 °C.

^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 1.35–1.61 (m, 4H), 1.77–1.89 (m, 1H), 1.93–2.05 (m, 1H), 3.14–3.29 (m, 2H), 4.28 (m, 1H), 4.90 (s, 1H), 5.10 (s, 2H), 6.76 (s, 1H), 7.29–7.40 (m, 5H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 21.56, 29.21, 30.98, 40.33, 57.58, 66.98, 128.10, 128.35, 128.69, 136.41, 152.84, 157.01, 170.24.

Ring-Opening Polymerization of Z-L-Lysine-*N*-Carboxyanhydride (ZLL-NCA)

To a solution of ZLL-NCA (306 mg, 1.0 mmol) in dry DMF (2.4 mL), *n*-$BuNH_2$/DMF solution (1.0 mL, 2.0 $\times$ 10^{-2} mmol/mL) was added. The polymerization was performed by stirring at room temperature for 24 h under argon atmosphere and precipitated in diethyl ether. The products were dried under vacuum to yield 242 mg (93%) of PZLL, as white powder, M_n: 12,500, M_w/M_n = 1.18.

^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 1.25–2.10 (br, 6H), 3.15 (s, 2H), 3.83 (s, 1H), 5.00 (s, 2H), 5.49 (s, 1H), 7.14–7.36 (br, 5H).

Deprotection of the Lysine Side Chain

The each PZLL from urethane derivatives or ZLL-NCA was deprotected by same procedure, to measure optical rotation and CD spectra in aqueous solution. To a solution of PZLL (70 mg) in trifluoroacetic acid (2 mL), 33% HBr solution in acetic acid (0.2 mL) was added. The reaction mixture was stirred for 2 h, at room temperature, and then poured into diethyl ether. After the precipitated polymers were collected by filtration, the polymer was washed with fresh diethyl ether twice and then dried under vacuum to yield 50 mg (89%) of PLL, as white powder.

^{1}H NMR (400 MHz, D_2O, δ, ppm): 1.23–1.48 (br, 2H), 1.53–1.78 (br, 4H), 2.92 (s, 2H), 4.24 (s, 1H).

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