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Well-Defined Homopolypeptides, Copolypeptides, and Hybrids of Poly(L-proline)

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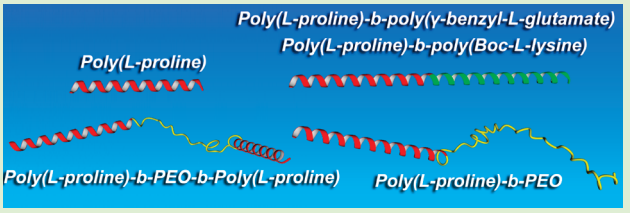
Supporting Information

ABSTRACT: L-Proline is the only, out of 20 essential, amino acid that contains a cyclized substituted α-amino group (is formally an imino acid), which restricts its conformational shape. The synthesis of well-defined homo- and copolymers of L-proline has been plagued either by the low purity of the monomer or the inability of most initiating species to polymerize the corresponding *N*-carboxy anhydride (NCA) because they require a hydrogen on the 3-N position of the five-member ring of the NCA, which is missing. Herein, highly pure L-proline NCA was synthesized by using the Boc-protected, rather than the free amino acid. The protection of the amine group as well as the efficient purification method utilized resulted in the synthesis of highly pure L-proline NCA. The high purity of the monomer and the use of an amino initiator, which does not require the presence of the 3-N hydrogen, led for the first time to well-defined poly(L-proline) (PLP) homopolymers, poly(ethylene oxide)-*b*-poly(L-proline), and poly(L-proline)-*b*-poly(ethylene oxide)-*b*-poly(L-proline) hybrids, along with poly(γ-benzyl-L-glutamate)-*b*-poly(L-proline) and poly(Boc-L-lysine)-*b*-poly(L-proline) copolypeptides. The combined characterization (NMR, FTIR, and MS) that results for the L-proline NCA revealed its high purity. In addition, all synthesized polymers exhibit high molecular and compositional homogeneity.



INTRODUCTION

Most polypeptides of α-amino acids form 3D structures through hydrogen bonding. Exceptions are the N-substituted polypeptides (NSP), which do not possess an amide (3-N) hydrogen, and their secondary structure is due to only the constraints induced in the main chain. The most characteristic is poly(L-proline) (PLP). PLP exhibits tertiary amide groups that lead to significant lowering of the barrier for cis−trans amide isomerization,[1] resulting in the formation of right-handed PLP I (cis conformation) and left-handed PLP II (trans conformation).[2] PLP II is adopted in water and organic acids,[3a,b] whereas PLP I is predominant in some organic solvents.[3c] PLP II is one of the most interesting homopolypeptides, first discovered in collagen,[4] because its conformation is highly extended with 3.1 Å axial translation[5] independent of pH and temperature up to 60 °C,[6] rendering it an ideal molecular ruler[7] or spacer with defined dimensions. However, recent studies have shown that individual PLP residues adopt the PLP I conformation even in water.[8] It has also been observed that the redox potential of the ferrocenyl group attached to the oligoproline chains depends on their length,[9] whereas PLP's rigid backbone has been used as a scaffold-linker for protein−protein recognition and cell penetration.[10]

It is well-documented[11] that high-molecular-weight poly(γ-benzyl-L-glutamate) (PBLG) forms helices, which are not considered long ideal tubes and present defects.[11] In contrast, low-molecular-weight PLP II is considered to be one of the most ideal and stiff helical polypeptidic chains with limited defects, although this has not been verified for well-defined high-molecular-weight PLPs. The most common route to high-molecular-weight polypeptide synthesis is via the polymerization of the α-amino acid *N*-carboxy anhydrides (NCAs). However, the living polymerization of NCAs was plagued, for several decades, by termination reactions.[12] In 1948, Astbury et al. initially remarked on the difficulty[13a] involved in the synthesis of poly-L-proline using NCAs, whereas at about the same time Waley and Watson[13b] carried out studies on the analogous N-substituted polysarcosine. Block and Bamford[14] attributed the controversy of various results of the polymerization of L-proline NCA (LP-NCA) to the high reactivity of LP-NCA to traces of water, whereas Goodman et al.[15] attributed the extensive racemization between equimolar quantities of LP-NCA and sodium methoxide to a proton abstraction from the α-carbon. Berger et al.[16] synthesized low-molecular-weight polypeptides of PLP, whereas the synthesis of high-molecular-weight PLP was reported[17] in the early 60s; however, the high-molecular-weight nature of the polypeptides was verified only via viscometry. Komoto et al. connected the two

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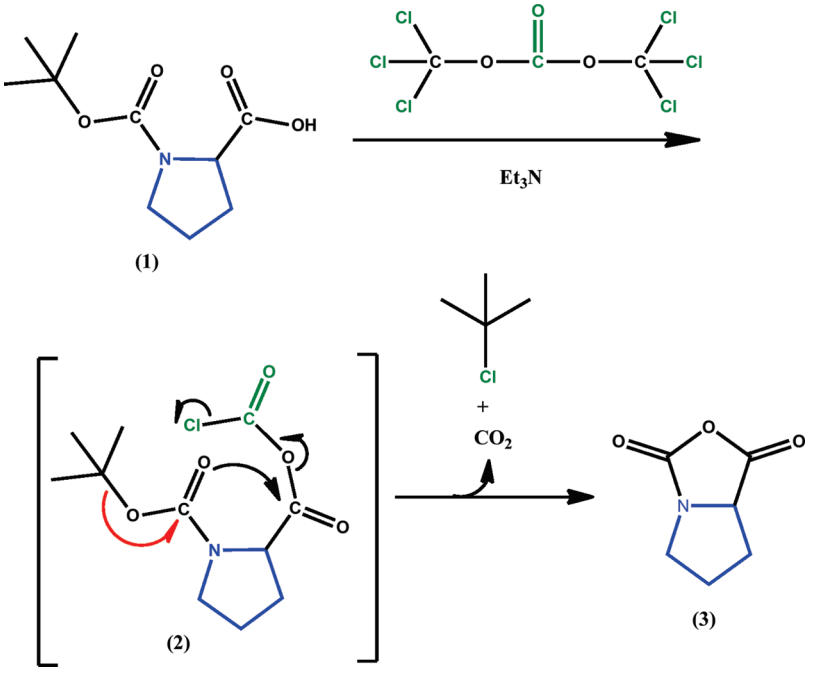
Scheme 1. Cyclization Reaction for the Synthesis of L-Proline *N*-Carboxy Anhydride



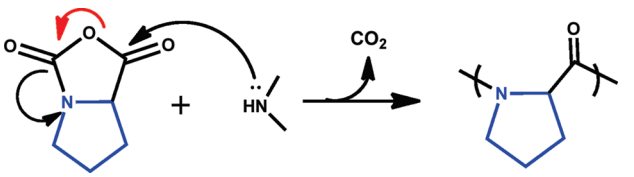
forms of PLP to the different crystallization behavior of the growing chains[18] during polymerization. Finally, Kricheldorf and coworkers[3c] determined the ratio of the two forms obtained during oligomerization of LP-NCA under different experimental conditions.

Recently, new initiating species have been developed for the living polymerization of NCAs. In the pioneer work of Deming et al.,[19a] followed later by Peng et al.,[19b] organometallic initiators were used, but these initiating systems could not polymerize LP-NCA because the polymerization mechanism requires the presence of the 3-N hydrogen. Silazane derivatives were used by Lu et al.,[20] but this mechanism also requires the presence of the 3-N hydrogen and consequently cannot be applied to LP-NCA. The Schlaad,[21a] Schué,[21b] and Zhang[21c] groups presented methodologies that suppressed the termination reactions, allowing for the polymerization of LP-NCA. We recently employed primary amines as initiators along with high vacuum techniques (HVTs) to create living conditions for the amino-initiated polymerization of NCAs.[22] Our approach is general and can also be applied for LP-NCA.

Several methodologies have been presented for the synthesis of LP-NCA.[16−18] The most common is the reaction of LP with phosgene or solid triphosgene, for the formation of the corresponding *N*-carbamoyl chloride (NCC), followed by the cyclization reaction in the presence of a base. Recently, polystyrene resins with different tertiary amines were used[23] to suppress the formation of side products. However, the purity of the synthesized LP-NCA was not verified by the synthesis PLPs.

We present here a novel methodology based on N-(Boc)[24] protected rather than neat L-proline, which leads, in high yields, to the synthesis of highly pure LP-NCA, as verified by NMR, FTIR, and MS. The high purity of the monomer in combination with an amino initiator and HVTs led to the synthesis of well-defined PLP homopolypeptides, the PLP hybrids poly(ethylene oxide)-*b*-poly(L-proline) (PEO-*b*-PLP), and PLP-*b*-PEO-*b*-PLP as well as the diblock copolypeptides PBLG-*b*-PLP and poly(*ε*-*tert*-butyloxycarbonyl-L-lysine)-*b*-poly(L-proline) (PBocLL-*b*-PLP).

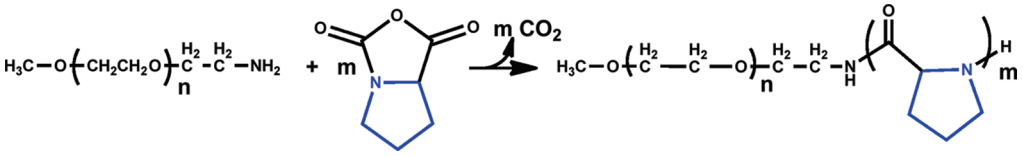
Scheme 2. Ring-Opening Polymerization of L-Proline *N*-Carboxy Anhydride



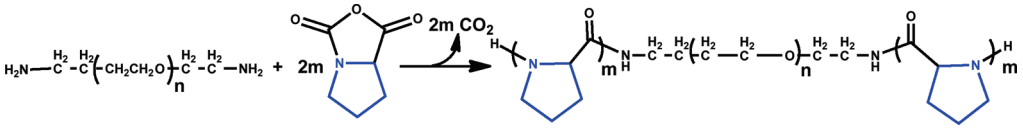
MATERIALS AND METHODS

Materials. Boc-Pro-OH (>99%) and triphosgene (99%) were purchased from Acros Organics. H-Pro-OH was purchased from Aldrich (>99%). Triethylamine (>99%, Acros Organics) was dried over calcium hydride for 1 day and then distilled and stored in the vacuum line over sodium. The appropriate quantity needed was freshly distilled in a vacuum line. Polystyrene resin bearing diethylamino groups (PS-DEAM) was purchased from Aldrich. Purification of THF (dried, max 0.005% water, Merck) was performed using standard HVTs reported elsewhere.[25] Ethyl acetate (>99.5%, Merck) was fractionally distilled over phosphorus pentoxide. Hexane (>99%, Merck) was fractionally distilled over sodium. Acetonitrile (ACN), the solvent for the synthesis of PLP (>99.5%, Merck), was left to react with phosphorus pentoxide for 1 day, degassed, and distilled under high vacuum to a previously flame-dried round-bottomed flask containing molecular sieves. ACN (LC-MS grade), the solvent for mass spectra experiments, was purchased from Labscan. Anhydrous dimethylamine (>99.9%, Aldrich, bp 7 °C) was condensed under high vacuum at −78 °C and was treated with sodium hydroxide pellets at room temperature for 1 day and was subsequently distilled into precalibrated ampules with break-seals. It was then diluted with ACN to the appropriate concentration in a sealed apparatus equipped with precalibrated ampules and kept away from light. End-functionalized

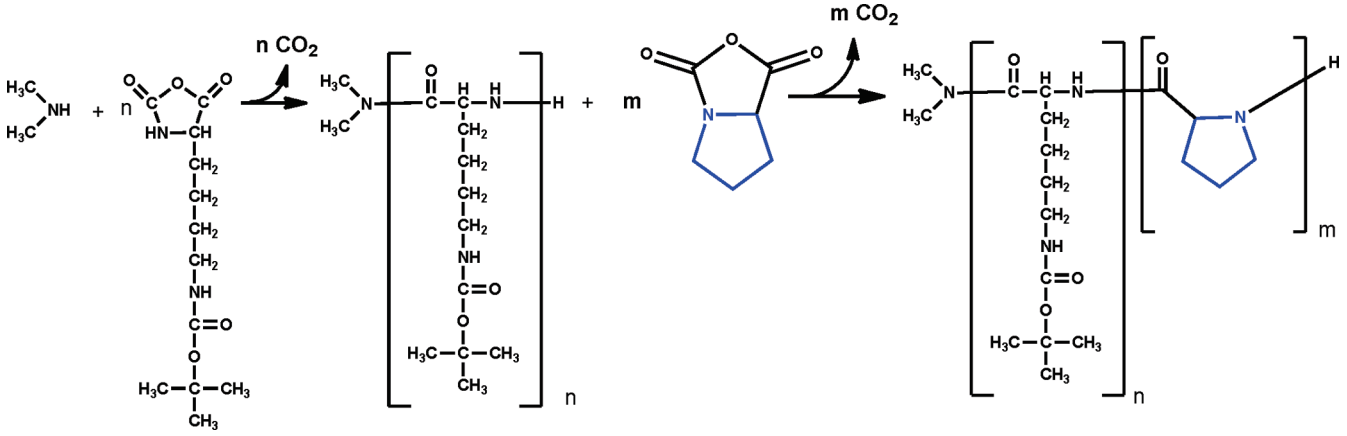
Scheme 3. Synthesis of Well-Defined PEO-*b*-PLP Hybrids



Scheme 4. Synthesis of Well-Defined PEO-*b*-PLP-*b*-PEO Hybrids



Scheme 5. Synthesis of Well-Defined PBocLL-*b*-PLP



monoamino poly(ethylene oxide) having molecular weights (10.0 and 20.0) $\times 10^3$ and α,ω-diamino-functionalized poly(ethylene oxide) having molecular weight 10.0×10^3 g/mol were purchased from Aldrich and were used as mono- and difunctional macroinitiators, respectively.

Size Exclusion Chromatography. Size exclusion chromatography (SEC) was used to determine the M_n and M_w/M_n values. The analysis was performed using two SEC sets. The one was composed of a Waters Breeze instrument equipped with a 2410 differential refractometer and a Precision PD 2020 two angles (15°, 90°) light scattering detector. The carrier solvent was a 0.1 M $NaNO_3$ solution of water/ACN (80/20 v/v) at a flow rate of 0.8 mL/min at 35 °C. Three linear Waters hydrogel columns were used. For PBLG and PBocLL homopolymers, the analysis was performed using a second SEC equipment. The system was composed of a Waters 600 high-pressure liquid chromatographic pump, Waters Ultrastyragel columns (HR-2, HR-4, HR-5E, and HR-6E), a Waters 410 differential refractometer detector, and a Precision PD 2020 two angles (15°, 90°) light scattering detector at 60 °C. A 0.1 N LiBr DMF solution was used as an eluent at a rate of 1 mL/min. For the water-soluble deprotected copolypetide PLL-*b*-PLP, the first SEC instrument was used, and the carrier solvent was 5% NaH_2PO_4 (w/v) solution of water/ACN (97/3 v/v) at a flow rate of 0.8 mL/min at 35 °C (pH 4).

NMR Spectroscopy. ^1H and ^{13}C NMR spectroscopy (400 and 75 MHz, respectively) were performed using a Bruker DPX 400 or a Varian Unity Plus 300/54 spectrometer. The spectra of the polymers were performed in CF_3COOD, whereas the spectra of the NCAs were taken in $CDCl_3$ at room temperature.

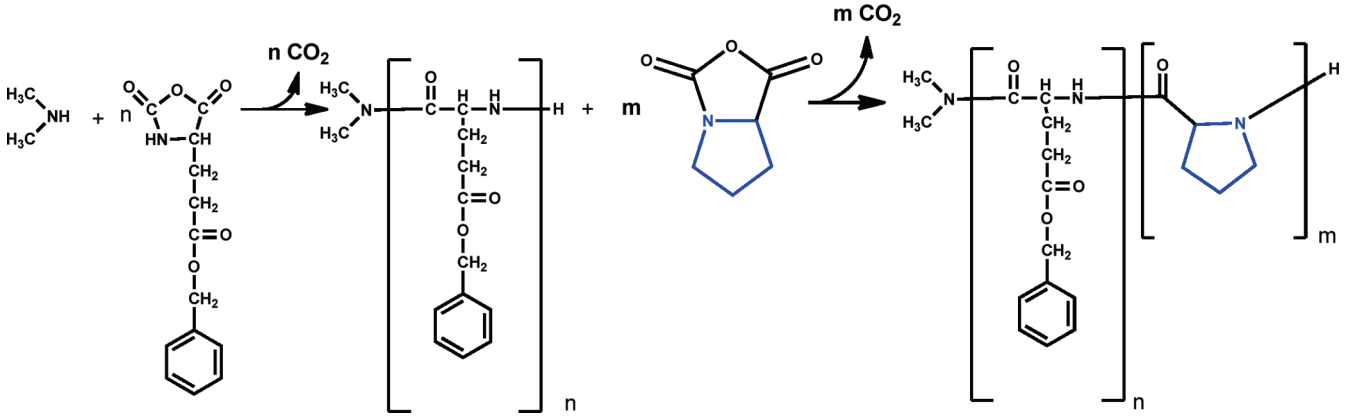
FTIR. FTIR measurements were performed with a Perkin-Elmer Spectrum One instrument in KBr pellets at room temperature in the range 450−4000 cm^{-1}.

Mass Spectrometry. Mass spectrometry was performed with a Thermo TSQ Quantum Access equipped with an IonMax electrospray (ESI) source. Purified L-proline-NCA was dissolved in ACN (LC-MS grade) and directly infused in the ESI source in positive ionization mode. Spray voltage ranged between 3000 and 4000 V; sheath gas and auxiliary gas pressures were maintained at 25 and 15 (a.u.), respectively, whereas two capillary temperatures were tested (150 and 270 °C). The full scan spectrum was recorded in a mass range of m/z 50−200 with a scan time of 0.5 s. Product ion scans and precursor ion scans were performed to verify that the observed ions belonged to the same molecule.

Circular Dichroism. Circular dichroism was performed with a Jasco J-815 model, featuring a peltier model PTC-423S/15 thermostabilizing system. The cell used was 1 mm Quartz Suprasil cell. The solutions of the PLP and hybrid polypeptides were made with Milli-Q water. Typical concentrations were $\sim 3 \times 10^{-4}$ g/mL.

X-ray Diffraction. X-ray powder diffraction (XRD) was carried out on a Rigaku Ultima IV diffractometer with a Cu Kα radiation (wavelength 1.54 Å) and was operated with $\theta/2\theta$ mode. The operating conditions of the diffractometer were 40 kV, 44 mA, and 1.76 kW, and data were collected at room temperature. The XRD data were collected at a 0.030 interval, and the scan speed was 0.5 deg/min. The technique used for measuring intensities was the focusing beam method (BB method).

Scheme 6. Synthesis of Well-Defined PBLG-*b*-PLP



Thermogravimetric Analysis. Thermogravimetric analysis (TGA) was performed using a TA Instruments TGA Q50 in nitrogen from 23 to 600 °C (10 °C/min).

Synthesis of L-Proline *N*-Carboxy Anhydride (LP-NCA). *tert*-Butyloxycarbonyl-L-proline (Boc-L-proline) (30 g, 0.139 mol) was dissolved in freshly distilled THF (600 mL) in a 1 L two-necked round-bottomed flask with a slight flow of dry Ar gas. Then, triphosgene (0.37 equiv, 15.2 g) was added under vigorous stirring. After 10 min, freshly distilled triethylamine (1.1 equiv, 21.3 mL) was added dropwise at 0 °C, leading to the formation of *N*-carboxy anhydride in a one-pot procedure with instantaneous precipitation of TEA·HCl salt. After stirring for 6.5 h at room temperature under Ar atmosphere, the reaction mixture was cooled at 0 °C to allow complete precipitation of the salt, and the precipitate was removed by filtration. The filtrate was then distilled under vacuum (to remove THF and excess phosgene), and the crude mixture was dried overnight in the high vacuum line.

Crude LP-NCA was then dissolved in ethyl acetate, chilled, and extracted[26] with ice-cold water (100 mL) until neutral pH. The organic phase was then separated, dried over $MgSO_4$, and filtered, and the solvent was distilled off in the vacuum line, yielding LP-NCA (8 g, yield ~50%).

Purification of LP-NCA. Crude LP-NCA was dissolved in the minimum amount of THF and a large excess of hexane (400 mL) was added, leading to a milky, thick precipitate which was cooled to −20 °C for complete precipitation. The solvent mixture was then removed under Ar filtration, using a microfilter candle for reversed filtration with a narrow tube (Duran, d = 13 mm, por.3), and the precipitate was dried on the high vacuum line. In this way, **1** (Scheme 1) was removed. The purification was monitored with FTIR and ^{1}H NMR spectroscopy.

The next step involved thermal dissolution of LP-NCA crystals in hexane. In this additional purification step, the dried crystals were suspended in a large amount of hexane (~ 500 mL) and gradually heated to 45 °C under vigorous stirring until the anhydride was thoroughly dissolved. The solution was then filtered immediately with a warm filter funnel (125 mL capacity, por.3) under vacuum in a new two-necked 1 L round-bottomed flask, whereas nonsoluble oily contaminants were removed. Very long needle-like white LP-NCA crystals were obtained when the flask was cooled to −20 °C. Cold hexane was filtered under Ar using a microfilter candle for reversed filtration, and the resulting LP-NCA crystals were dried under high vacuum. This procedure resulted to removal of **2** of Scheme 1. The procedure was repeated if contamination was indicated by FTIR spectroscopy. The purity of the final product was verified by FTIR and ^{1}H and ^{13}C NMR spectroscopy, and electrospray ionization mass spectroscopy (ESI-MS), and it was stored in a glovebox. Overall yield: 6 g (30%) m.p.: 53−55 °C.

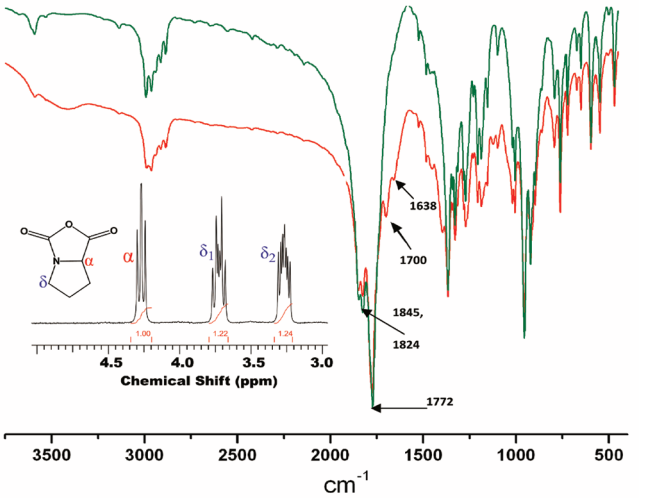


Figure 1. FTIR of the crude product (red line) and of the purified LP-NCA (green line) along with the ^{1}H NMR (inset) spectra of LP-NCA.

LP-NCA. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.27 (dd, J = 8.78, 7.84 Hz, 1H), 3.71 (dt, J = 11.4, 7.47, 7.47 Hz, 1H), 3.26 (ddd, J = 11.4, 8.32, 4.92 Hz, 1H), 2.25 (m, 1H), 2.11 (m, 2H), 1.88 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.0, 155.2, 63.3, 46.8, 28.0, 27.2. IR (thin film) 2994, 2958, 1845, 1824, 1772, 1364, 1328, 1270, 1202, 1185, 1150, 1095, 998, 952, 916 cm^{-1}. ESI-MS m/z 70, 114, 130, 141 (capillary temperature 150 °C, spray voltage 3000−4000 V).

Synthesis of Polypeptides. All manipulations were performed under high vacuum in glass reactors and equipped with break seals, glass-covered magnets, and constrictions for the addition of reagents and removal of the intermediate products following well-established HVTs.[25]

Synthesis of Well-Defined Poly(L-proline). The reaction used for the synthesis of well-defined PLP is given in Scheme 2.

LP-NCA (0.67 g) was pumped for 1 day to dryness in the high vacuum line in a specially designed glass reactor (equipped with break-seal along with a glass-covered magnet), which had been previously flame-dried several times. The monomer was then dissolved in freshly distilled ACN, and the glass magnet of the initiator ampule was subsequently ruptured, allowing the solution of dimethylamine (5×10^{-5} mol, A/I = 94, for PLP no. 2) to react with the monomer under vigorous stirring. After a few minutes, the solution became turbid, and the heterogeneous polymerization induced was left for 1 week, occasionally being degassed to remove the generated CO_2. The final polypeptide (mainly PLP I configuration)

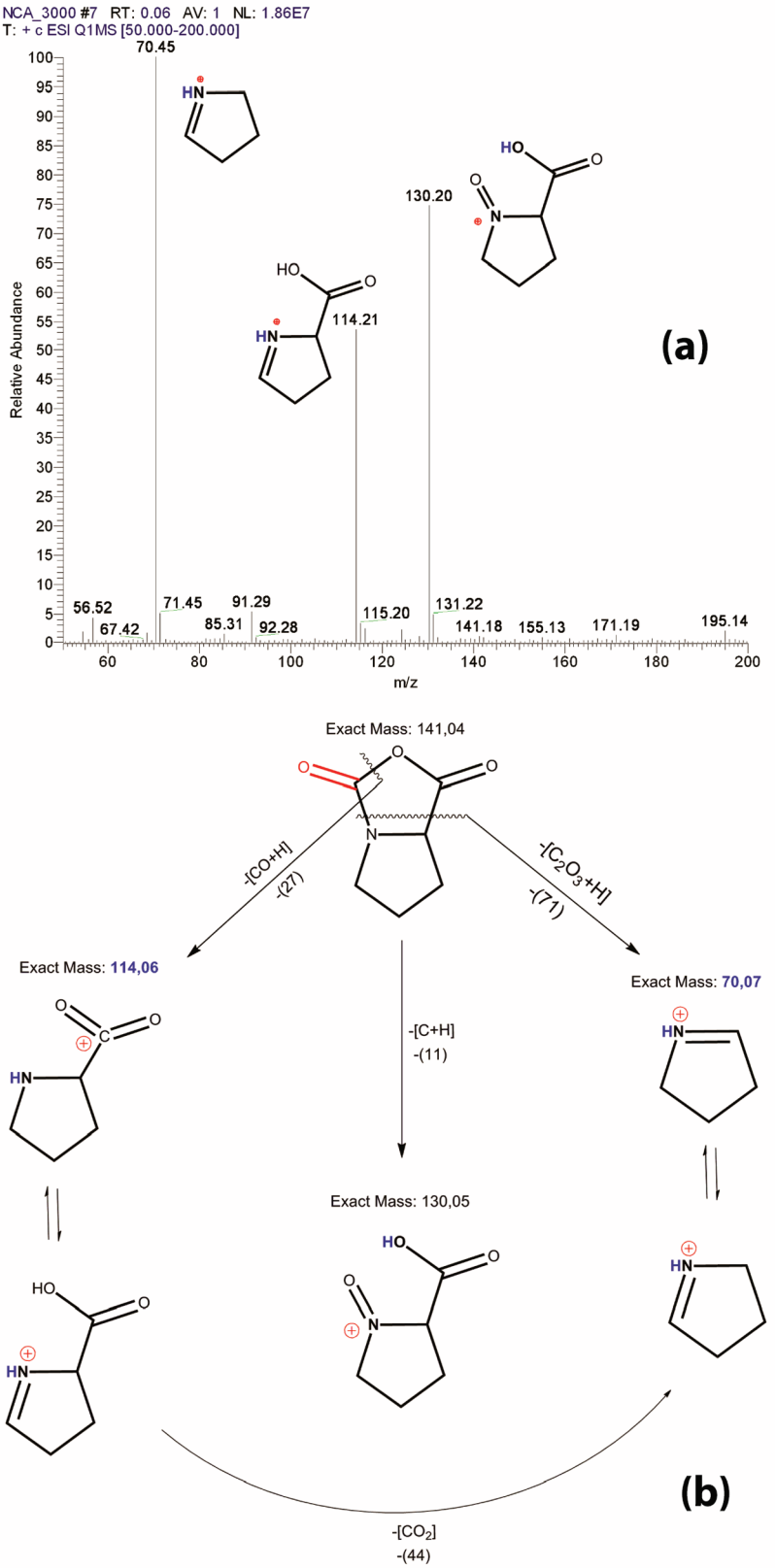
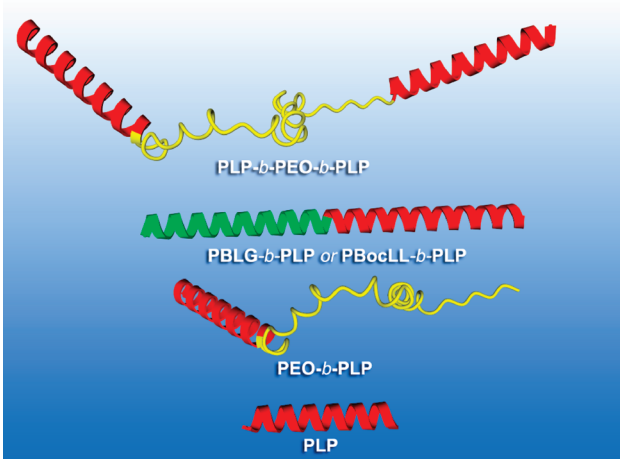


Figure 2. (a) Positive electrospray mass spectrum (+ESI-MS) spectra of purified LP-NCA. Capillary temperature 150 °C, spray voltage 3000 V. (b) Fragmentation pattern of LP-NCA.

was precipitated in cold ether and dried in vacuo. The purity of the product was verified by SEC-TALLS, FTIR, ^{1}H NMR, and ^{13}C NMR.

To obtain the water-soluble poly-L-proline II (PLPII) form, the polypeptide was suspended in Milli-Q water (2×10^{-3} g/mL) for

Scheme 7. Homopolypeptides, Copolypeptides, and Hybrids of Poly(L-proline) Synthesized



1 day under vigorous stirring to become soluble and centrifuged, and the water-soluble part was freeze-dried. The conversion was about 60−80%, in agreement with previous studies.[16]

PLP II. ^{1}H NMR (300 MHz, CF_3COOD) δ 4.76 (1H, C^{α}), 3.78 (1H, $C^{\delta 2}$), 3.58 (1H, $C^{\delta 1}$), 2.29 (1H, $C^{\beta 2}$), 1.99 (3H,, $C^{\beta 1+\gamma}$). ^{13}C NMR (75 MHz, CF_3COOD): δ 175.1, 62.5, 50.9, 30.3, 27.0. IR (thin film) 3450, 2962, 2879, 1640, 1426, 1328, 1259, 1202, 1163, 1092, 1040, 916 cm^{-1}. GPC (0.1 M $NaNO_3$) water/ACN 80:20 at 35 °C, M_w/M_n = 1.23.

Synthesis of Poly(ethylene oxide)-*b*-poly(L-proline) (PEO-*b*-PLP). The reaction used for the synthesis of well-defined PEO-*b*-PLP is given in Scheme 3.

Amino-end monofunctional PEO having molecular weights of either 10.0 or 20.0 × 10^3 g/mol was pumped to dryness in the high vacuum line in a custom-made 100 mL glass apparatus (equipped with a filter), which had been previously flame-dried several times. Benzene was then distilled to remove azeotropically traces of water, the solvent was distilled off, and the polymer was left to dryness overnight. ACN was distilled to dissolve the polymer, which was finally filtrated in an ampule and used as a macroinitiator. The heterogeneous polymerization procedure is the same as that presented for poly-L-proline in ACN. To transform the PLP block to its PLP II form, the hybrid polypeptide was suspended in Milli-Q water for 1 day under vigorous stirring, centrifuged, and then was freeze-dried.

PEO-b-PLP No. 1. ^{1}H NMR (300 MHz, CF_3COOD) δ 4.70 (1H, C^{α}), 4.15−3.15 (4H, $-CH_2CH_2O$ and 2H, C^{δ}), 2.24 (1H, $C^{\beta 2}$), 1.93 (3H, $C^{\beta 1+\gamma}$). GPC (0.1 M $NaNO_3$) water/ACN 80:20 at 35 °C, M_w/M_n = 1.10.

Synthesis of Poly(L-proline)-*b*-poly(ethylene oxide)-*b*-poly(L-proline) (PLP-*b*-PEO-*b*-PLP). The reaction used for the synthesis of well-defined PEO-*b*-PLP-*b*-PEO is given in Scheme 4.

The synthetic procedure was the same as that in the previous case except that the α,ω-diamino PEO having molecular weight of 10.0 × 10^3 g/mol was used.

PLP-b-PEO-b-PLP No. 2. ^{1}H NMR (300 MHz, CF_3COOD) δ 4.73 (2H, C^{α}), 3.85−3.35 (4H, $-CH_2CH_2O$ and 4H, C^{δ}), 2.26 (2H, $C^{\beta 2}$), 1.96 (6H, C $C^{\beta 1+\gamma}$). GPC (0.1 M $NaNO_3$) water/ACN 80:20 at 35 °C, M_w/M_n = 1.09.

Synthesis of Poly(ε-*tert*-butyloxycarbonyl-L-lysine)-*b*-poly-(L-proline) (PBocLL-*b*-PLP). The reactions used for the synthesis of the PBocLL-*b*-PLP are given in Scheme 5.

ε-*tert*-Butyloxycarbonyl-L-lysine-NCA was synthesized according to previously reported method.[27] Boc-L-Lys-NCA (1.2 g) was pumped for 1 day to dryness in the high vacuum line in a specially designed glass reactor (equipped with break-seal and glass-covered magnet), which had been previously flame-dried several times. The monomer was then dissolved in freshly distilled DMF, and the glass magnet of the initiator ampule was subsequently ruptured, allowing the solution of dimethylamine (0.95 × 10^{-5} mol, A/I = 462) to react with the monomer under vigorous stirring for 3 days, with occasional degassing. An aliquot of the solution was removed after the completion of the polymerization of ε-*tert*-butyloxycarbonyl-L-lysine-NCA for characterization. Then, LP-NCA solution in DMF was cannula transferred (in an Ar/vacuum line) in the specially designed glass reactor containing the PBocLL macroinitiator, transferred to the high vacuum line, degassed, and left 1 week under vigorous stirring for the polymerization of the PLP branch. The polymerization was heterogeneous, and the final copolypeptide was precipitated in cold ether and dried in vacuo.

To obtain the water-soluble PLL-*b*-PLP, the polypeptide was suspended in TFA and left to react until complete dissolution. Then, the organic acid was distilled in the high vacuum line, and the product was dissolved in Milli-Q water.

PBocLys. GPC (0.1 M LiBr) DMF at 65 °C, M_n = 99.1 K, M_w/M_n = 1.18.

PLL-b-PLP. ^{1}H NMR (300 MHz, CF_3COOD) δ 5.25−4.35 (1H, C^{α} of PLP and 1H, C^{a} of PLL), 4.20−3.50 (2H, C^{δ}), 3.20 (2H, C^{e}), 2.75−1.1 (4H, $C^{\beta+\gamma}$ of PLP and 6H, C^{b+c+d} of PLL). GPC (5% NaH_2PO_4) water/ACN 97:3 at 35 °C, M_w/M_n = 1.10.

Synthesis of Poly(γ-benzyl-L-glutamate)-*b*-poly(L-proline) (PBLG-*b*-PLP). The reactions used for the synthesis of the PBLG-*b*-PLP are given in Scheme 6.

The procedure followed was the same as that in the case of PBocLL-*b*-PLP, the only difference being that the LP-NCA solution that was transferred with the cannula (in the Ar/vacuum line) was in THF and the polymerization of the PLP branch was nearly homogeneous. The final product was insoluble in DMF, and no SEC could be obtained. TGA measurements were carried out for the two samples to determine qualitatively the composition of the two blocks.

PBLG No. 1. GPC (0.1 M LiBr) DMF at 65 °C, M_n = 20 K, M_w/M_n = 1.02.

PBLG-b-PLP No. 1. TGA (range of temperature: 23−600 °C, flow gas: N_2) 215−360 °C for PBLG, 360−450 °C for PLP.

RESULTS AND DISCUSSION

Synthesis of the Monomer. The most critical factor in the synthesis of well-defined PLP polymers is the purity of the monomer.

In an early attempt, LP was reacted with triphosgene (details of the experimental procedure are given in the Supporting Information), followed by reaction of the resulting NCC with an equimolar amount of triethylamine for the cyclization reaction at room temperature, at 0 °C, or at −25 °C, according to literature.[16−18] In all cases, the LP-NCA yield was low (Supporting Information, Table 1), and the main side products were crystalline diketopiperazine (DKP), along with NCC, which is oily and soluble in THF/hexane and can be effectively removed by recrystallizations. However, after this procedure, the product was enriched in DKP (Supporting Information, Scheme S1) because it also crystallizes in hexane. The presence of DKP was monitored by FTIR at 1654 cm^{-1} (DKP characteristic peak) (Supporting Information, Figure F1). The synthetic approach that uses polystyrene resins bearing diethylamino groups (PS-DEAM) was also followed (details of the experimental procedure are given in Supporting Information); however, polymeric side products were obtained (Supporting Information, Figure F2) at 1640 cm^{-1}, and the LP-NCA produced was not stable over time

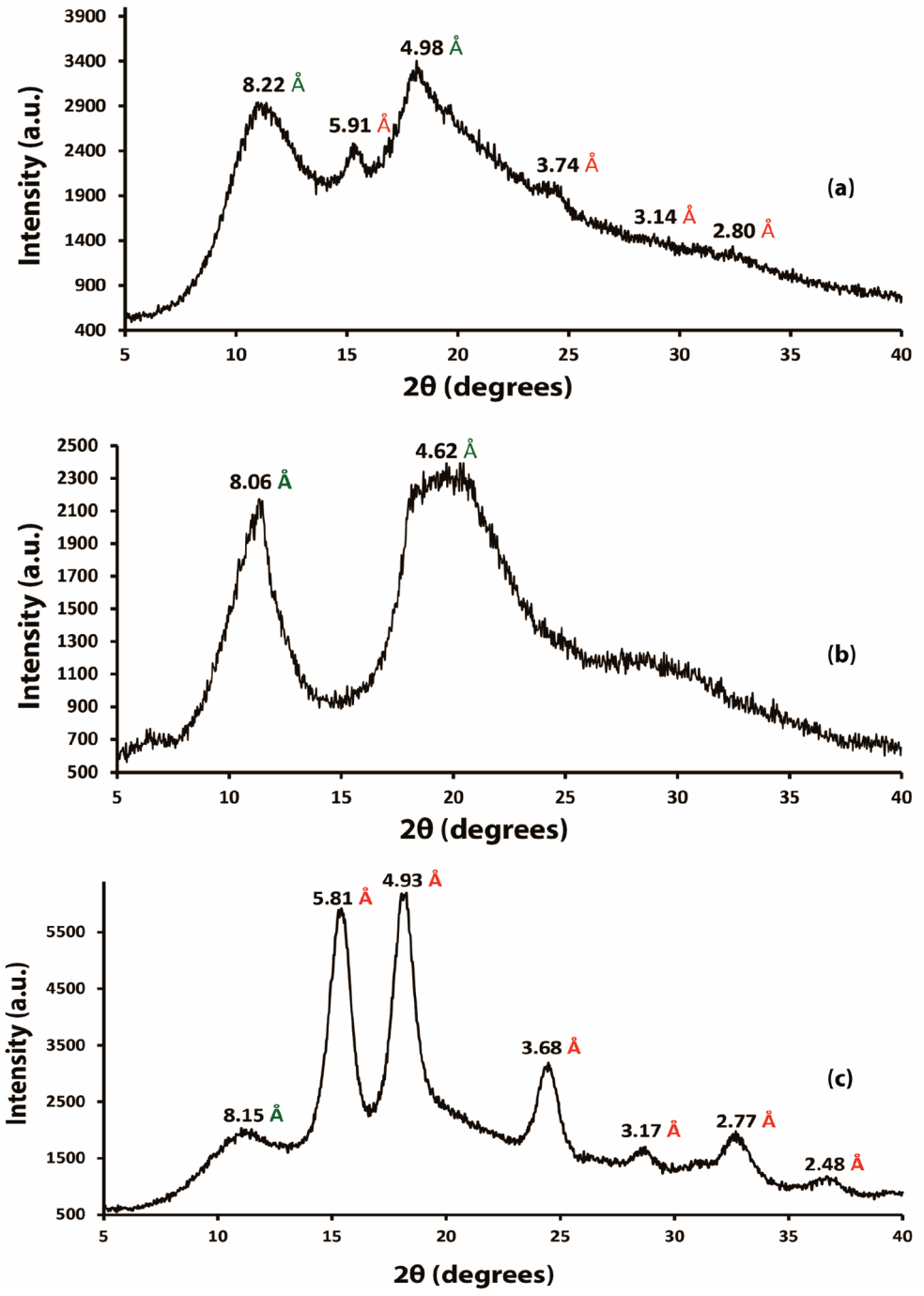


Figure 3. (a) XRD powder pattern of PLP no. 2 after precipitation, showing that is mainly PLP I form. (b) XRD powder pattern of PLP I no. 3 from acetic acid/PrOH 1:9. (c) XRD powder pattern of PLP II no. 3 from formic acid.

(Supporting Information, Table 1). The above methods, therefore, were not appropriate for highly pure LP-NCA.

It is well-documented that DKP formation is not favored if the amine group is protected.[28] Moreover, polymeric side products can be formed when secondary cyclic unprotected amines are present in the synthesis of NCA. We hypothesized that N-(Boc)-protected proline (**1**, Scheme 1) would block the formation of side products. The synthesis of LP-NCA was a one-pot procedure involving the formation of the carbonyl chlorocarbonate (**2**, Scheme 1) as an intermediate, followed by the cyclization reaction. After filtration, the resulting LP-NCA was formed in high yield. Approximately 5% of each **1** and **2** remained unreacted. LP-NCA was also contaminated with traces of the $Et_3N \cdot HCl$ salt and excess phosgene, which were removed in the aqueous phase by extraction with chilled ethyl acetate/water mixture. The unreacted species were removed by recrystallization in THF/hexane (removal of unreacted **1**), followed by dissolving the LP-NCA in warm hexane (45 °C) and filtration (removal of the side product **2**). LP-NCA purity was monitored by NMR (Supporting Information,

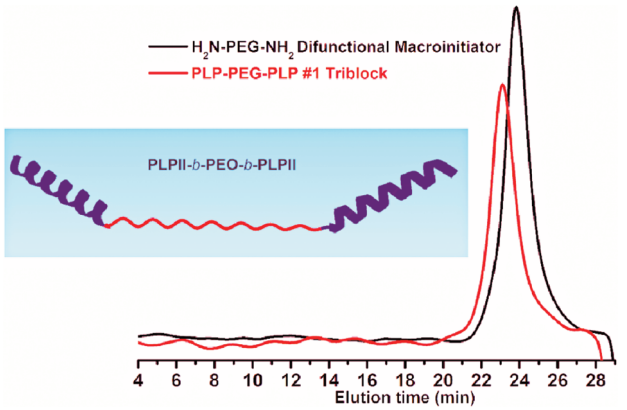


Figure 4. SEC chromatograms of the difunctional macroinitiator H_2N-PEO-NH_2 and the PLP-*b*-PEO-*b*-PLP no. 1 in water.

Figures F3 and F4), FTIR (Figure 1), and ESI-MS (Figure 2) spectroscopies.

The characteristic signal of the LP-NCA α proton at 4.27 ppm is present in the ^{1}H NMR spectrum (inset of Figure 1), whereas that of **2** appears at 4.59 and 4.48 ppm and that of **1** appears at 4.22 and 4.32 ppm. The high purity of the LP-NCA is confirmed by the lack of peaks corresponding to side products. The FTIR spectra of the crude product (red line) and the purified LP-NCA (green line) are provided in Figure 1. The C═O stretch of the carbonyl chlorocarbonate (**2**) appears at 1700 cm^{-1}, whereas that of **1** appears at 1638 cm^{-1}. The lack of a peak in the 1740−1638 cm^{-1} range verifies the absence of side materials.

Compared with FTIR and NMR spectroscopies, ESI-MS can detect smaller quantities of impurities. The (+)ESI-MS of the purified LP-NCA at a spray voltage of 3000 V produced three intense peaks at *m*/*z* 70, 114, and 130 (fragments of the same molecule), with the absence of any impurity ions (Figure 2a,b). The molecular ion (*m*/*z* 141), as well as the adducts (+41) with ACN of the observed ions (*m*/*z* 155 and 171), are also shown in Figure 2a. The observed ions were sufficiently explained by the fragmentation pattern proposed in Figure 2b. When the spray voltage gradually increased, the peak of *m*/*z* 130 decreased and disappeared at 4000 V, with a simultaneous increase in the other two main ions (*m*/*z* 114 and 70). To verify that the three main ions were part of the same molecule, product ion scans of *m*/*z* 114 and 130 were performed at collision energies of 10, 15, and 20 eV. In both cases, the only product was *m*/*z* 70. In an additional experiment, precursor ion scans of product ion *m*/*z* 70 revealed two ions at *m*/*z* 114 and 140, verifying that all of these ions are produced from the molecular ion of L-proline-NCA (*m*/*z* 141) (Supporting Information, Figure F5). This is, to our knowledge, the first report confirming the high purity of LP-NCA (Supporting Information, Scheme S2) with three spectroscopic methods.

The high purity of the synthesized LP-NCA has been achieved by: (a) the use of N-protected proline rather than the unprotected one, which on one hand prevents the formation of DKP and on the other hand results in Boc-protected byproduct with the appropriate solubility for easy removal, and (b) the efficient purification methodology used, which quantitatively removed the side products.

Synthesis of the Polymers. The synthesized polymers are shown in Scheme 7.

PLP Homopolymers. A series of water-soluble PLPs with rather narrow distributions was synthesized by heterogeneous polymerization of highly pure LP-NCA in ACN, using dimethylamine as initiator and HVT. The use of HVT ensures the effective purification of chemicals and the successful preparation of well-defined polypeptides. The polymerization yield was ∼100%, the molecular weights close to that expected from stoichiometry, and the polydispersity indices (*I*) were surprisingly as low as 1.26 (Supporting Information, Figure F6). It was possible to obtain PLP homopolymers only up to 13.0×10^3 g/mol because of the heterogeneity of the polymerization. In the case of the copolymers, the synthesis of higher-molecular-weight PLPs (140.0×10^3 g/mol) was possible. The molecular characteristics of the homopolypeptides are shown in Table 2 (Supporting Information). The PLPs were characterized by GPC, ^{1}H NMR, ^{13}C NMR, and CD in water (Supporting Information, Figures F6, F7 and F8), whereas comparisons between the two forms were made in the solid state with IR and XRD.

The PLP II form was also identified in water by circular dichroism, showing a strong negative band[29] at 206 nm and a weak positive band at 228 nm (Supporting Information, Figure F9). This left-handed helix is a common secondary structure in natural proteins (gelatin, elastin, casein, collagen).

The two forms of PLP can be identified by FTIR and XRD in the solid state. By FTIR, the identification of PLP I is confirmed by the peaks at 960, 1355, and 1155 cm^{-1}, whereas these peaks are significantly reduced in intensity or completely eliminated in the PLP II form (Supporting Information, Figure F10). According to XRD data, the PLP II form gives multiple peaks because of its well-organized macromolecular structure, whereas the PLP I form with low crystallinity gives only characteristic peaks at 8.06 and 4.62 Å. As it is identified by FTIR and XRD measurements, PLP is mainly form I after precipitation (Figure 3a). To adopt the fully cis PLP I, a solvent mixture of acetic acid/PrOH 1:9 was used[30,31] (Figure 3b), whereas for the fully trans PLP II form, water or organic acids were used (Figure 3c). The transition between the two forms is cooperative and reversible. Peaks at 5.81, 4.93, 3.68, 3.17, 2.77, and 2.48 Å are characteristic of form II, confirming previous XRD studies.[5b,18,31] Our XRD measurements, however, reveal that even after treatment with formic acid (and centrifugation), a small fraction of cis-residues is still present (Figure 3c), as proved by the peak at 8.15 Å. This is in accordance with previous studies,[8] where a small fraction of cis prolines in PLP II oligoprolines was found. This was predicted in a theoretical study by Schimmel and Flory,[32] who pointed out that even a small fraction of cis residues should be present in PLP II, which would lower the end-to-end distance of the highly organized chain.

Synthesis of PEO-*b*-PLP and PLP-*b*-PEO-*b*-PLP Hybrids. Diblock and triblock water-soluble hybrid polypeptides containing PLP blocks were also synthesized in ACN using amino-terminated and α,ω-diamino poly(ethylene oxide) as difunctional macroinitiators. Initially, polymerization was homogeneous because of the solubility of PEO in ACN, but when the molecular weight of PLP increased, the polymerization became heterogeneous. From the SEC trace of PLP-*b*-PEO-*b*-PLP no. 1 (Figure 4), it is obvious that the polydispersity index (1.10) is similar to that of the α,ω-diamino precursor, indicating that the higher polydispersity of the PLP homopolypeptides was due to the heterogeneity of the polymerization. The molecular characteristics of the hybrid polypeptides were those expected from stoichiometry (Supporting Information, Table 2). The composition was verified by ^{1}H NMR spectroscopy in CF_3COOD (Supporting Information, Figure F11 and F12). Jeon et al.[33]

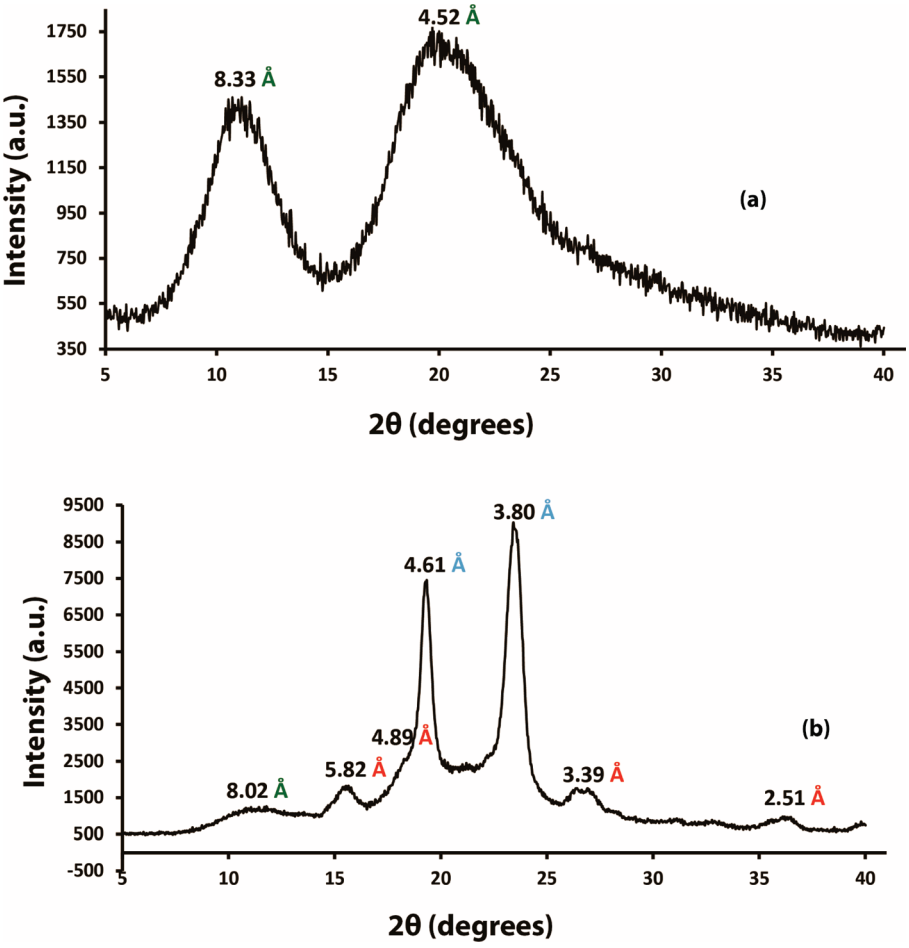


Figure 5. (a) XRD powder pattern of PEO-*b*-PLP no. 1 directly after the synthesis without any treatment. (b) XRD powder pattern of PLP-*b*-PEO-*b*-PLP no. 1 directly after the synthesis without any treatment.

synthesized a similar triblock; however, they reported that PEO remained unreacted and no SEC was given. The diblock synthesized here is reported for the first time.

The XRD data reveal two different pictures for the conformation of diblock and the triblock hybrid polypeptides after precipitation. In the PEO-*b*-PLP no. 1, the fully cis PLP I form is observed and PEO cannot crystallize, implying an interfacial mixing (Figure 5a). However, after dissolution in formic acid, it mutarotates to PLP II. The PLP-*b*-PEO-*b*-PLP no. 1 adopts mainly the PLP II form after precipitation, and the crystallinity of PEO is observable (Figure 5b). Dissolution in formic acid does not change the spectra. The diblock no. 1 and triblock no. 1 have different macromolecular architectures as well as different PLP contents but the same size PEO block. From the data in Figure 5a,b, it seems that the conformations of both the formed PLP and the PEO crystallinity are affected by the macromolecular architecture and the PLP/PEO composition.

CD measurements of both the diblock and triblock hybrid polypeptides in water reveal that after treatment in water, the PLP II form is adopted (Figure 6). Despite a slight shift of the peaks of the PLP II form in the hybrid triblock (205 and 227 nm), the spectrum is still indicative of a left-handed helix.

Synthesis of PBLG-*b*-PLP and PBocLL-*b*-PLP Copolypeptides. Block copolypeptides of L-proline were synthesized using the sequential addition methodology. Boc-L-Lys NCA or

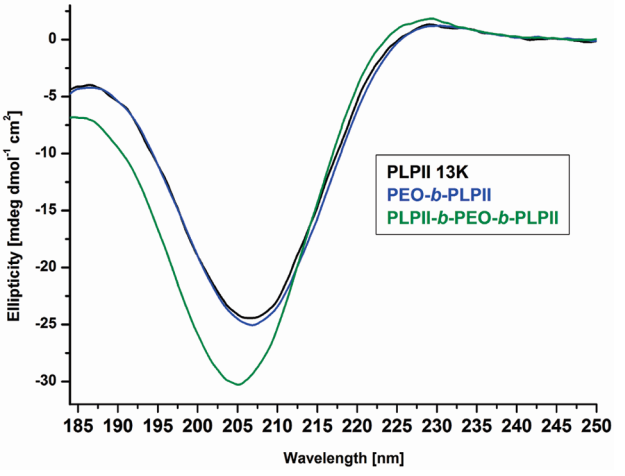


Figure 6. CD spectra of hybrid polypeptides in water.

Bn-L-Glu NCA were both polymerized first in DMF using dimethylamine as the initiator, with subsequent addition of the LP-NCA solution, leading to the polymerization of the second block. The protected copolypeptides formed aggregates in all solvents. In organic solvents such as DMF, the PBocLL and PBLG were soluble, whereas the PLP block was not. In water, the

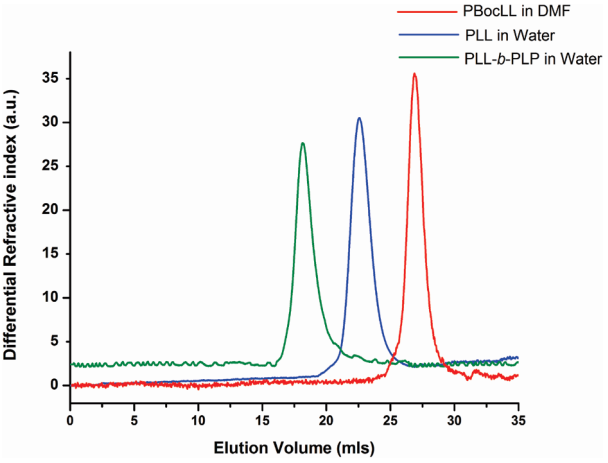


Figure 7. SEC chromatograms of the PBocLL in 0.1 N LiBr DMF solution (red line), the corresponding deprotected PLL in 5% NaH_2PO_4 (w/v) solution of water/ACN (97/3 v/v) (blue line), and the diblock PLL-*b*-PLP II in the same eluent system (green line).

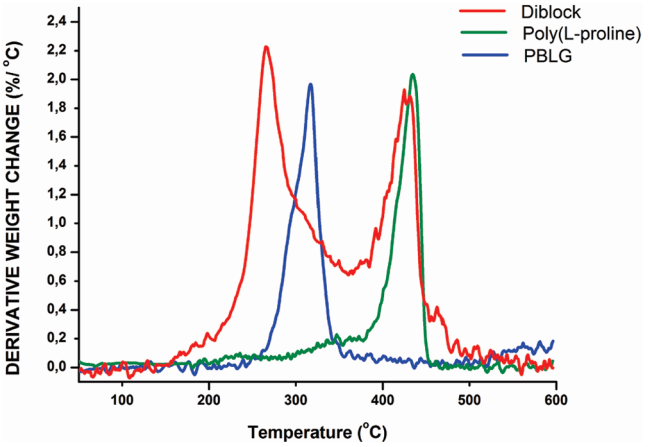


Figure 8. TGA spectra of PBLG-*b*-PLP no. 1.

PBLG and PBocLL were insoluble, whereas the PLP became soluble after it was transformed to the PLP II form. The molecular weight and the polydispersity indices of the first blocks, that is, PBLG and PBocLL, were obtained by SEC-TALLS in DMF/LiBr, revealing their high molecular homogeneity and the molecular weights expected from stoichiometry.

In the case of PBocLL-*b*-PLP, the final copolypeptide was dissolved in TFA, resulting in the deprotection of PBocLys, simultaneously leading to the transformation to the PLP II form, thus forming the water-soluble PLL-*b*-PLP II. The composition of the deprotected copolypetide was obtained by NMR spectroscopy (Supporting Information, Figure F13). To obtain the molecular weight and the polydispersity of the PBocLL-*b*-PLP copolypeptide, the corresponding water-soluble PLL-*b*-PLP II was characterized by SEC-TALLS (Figure 7).

The molecular weight of the PLP block of the PBocLL-*b*-PLP copolypeptide was obtained as follows: The molecular weight of the protected PBocLL precursor was 99.1×10^3 g/mol obtained by SEC-TALLS in DMF, whereas the molecular weight of corresponding deprotected block was 68.0×10^3 g/mol, obtained in water/ACN system (pH 4). The total molecular weight of the water-soluble PLL-*b*-PLP II copolypeptide was 208.0×10^3 g/mol. Therefore, the molecular weight of PLP block is the resulting difference, 140.0×10^3 g/mol. It is obvious from Figure 7 that even if the PLP block is heterogeneously polymerized, the final copolypeptide exhibits low polydispersity and has the molecular weight expected from stoichiometry. Therefore, a high-molecular-weight PLP (M_n = 140.0×10^3 g/mol) was obtained, reported for the first time.

The composition of the PBLG-*b*-PLP copolypeptides was determined by TGA measurements. PBLG decomposes between 265 and 330 °C and leaves a residue of ~20%, whereas PLP decomposes at significantly higher temperatures, between 380 and 450 °C, leaving the same amount of residue. This difference in decomposition temperature allowed a qualitative estimation of the composition. In the case of PBLG-*b*-PLP no. 1, the first transition between 215 and 360 °C is due to PBLG decomposition, whereas the second transition between 360 and 450 °C is due to that of PLP (Figure 8). In the case of PBLG-*b*-PLP no. 2, the first transition between 250 and 350 °C is due to PBLG decomposition, whereas the second transition between 325 and 450 °C is due to that of PLP. The total molecular weight of the PBLG-*b*-PLP given in Table 2 of the Supporting Information is that expected from stoichiometry. The obtained compositions of these copolypeptides are in excellent agreement with those expected from stoichiometry. It was not possible to measure the polydispersity indices and the total molecular weight of the PBLG-*b*-PLP copolypeptides by SEC because of the difference in solubility of the two blocks. However, because the analysis of the PBocLL-*b*-PLP showed the high degree of molecular and compositional homogeneity of the copolypeptides and because the PBLG-*b*-PLPs were similarly synthesized, we can conclude that these copolypeptides are also well-defined.

CONCLUSIONS

For many years, the synthesis of PLP homo- and copolymers has been plagued by the inability to synthesize pure monomer and to employ appropriate initiating systems. Our synthetic approach led to highly pure LP-NCA due to: (a) the use of N-protected proline rather than the unprotected one, which on one hand prevents the formation of DKP and on the other hand results in Boc-protected byproduct having the appropriate solubility for easy removal, and (b) the efficient purification methodology used, which quantitatively removed the side products. This novel route to highly pure LP-NCA, in combination with an amine initiator and HVT, opens new avenues for the synthesis of well-defined PLPs and PLP-containing polypeptides. The synthesis of amphiphiles containing the PLP II, a hydrophilic helix independent of pH and temperature within the physiological range, is expected to have significant applications on the formation of nanoconstructs for drug and gene delivery. The synthesis of poly(hydroxyl-L-proline)-based materials will be presented in a forthcoming paper.

ASSOCIATED CONTENT

Supporting Information. Attempts leading to impure LP-NCA, composition of crude products from the various synthetic methods of LP-NCA, monitoring the purity of LP-NCA by NMR and IR spectroscopy, molecular characteristics of the synthesized polymers, SEC chromatogram of PLP homopolypeptides, circular dichroism of poly(L-proline) as a function of temperature, IR spectra of PLP I and II, NMR spectra of PLP,

hybrids and polypeptidic samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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