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ORIGINAL PAPER

Formation of carbamates and cross-linking of microbial poly(ε-L-lysine) studied by ^{13}C and ^{15}N solid-state NMR

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Abstract Formation of carbamates by amino groups of poly(ε-L-lysine) (ε-PL) and cross-linking of ε-PL were studied by using ^{13}C and ^{15}N solid-state NMR. It is a characteristic found in ε-PL cast from basic aqueous solution exposed to the air or gaseous CO_2. It is not observed in ε-PL cast from acidic aqueous solution and ε-PL cast from degassed aqueous solution under CO_2 free environment. The carboxyl carbon and amide nitrogen appear at 164 ppm in ^{13}C spectrum and 92 ppm in ^{15}N spectrum, respectively, which arise when some amino groups of ε-PL react with gaseous CO_2 to make carbamates. In addition to these peaks a peak at 171 ppm appears. We assigned it to amide C=O carbons which can not make intermolecular hydrogen bondings since there exist bulky carbamates groups close to these C=O groups. Self-assembly of ionic pairs of ammonium groups and carbamate anions leads to cross-linking of ε-PL.

Keywords Poly(ε-L-lysine) Solid-state NMR · Microbial polymer · Carbamates · Cross-linking

Introduction

Biopolymers such as poly(amino acid)s have attracted more attention and interest to substitute synthetic polymers with similar characteristics due to their low environmental impacts. Poly(ε-L-lysine) (ε-PL) (Scheme 1) is one of a few poly(amino acid)s which are known to occur in nature [1–5]. Microbial ε-PL is a

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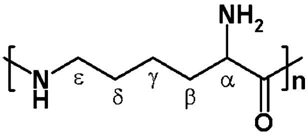

Scheme 1 Repeating units of poly(ε-L-lysine) (ε-PL)

product of a variant of *Streptomyces albulus*. In ε-PL molecules, the ε-amino group of a L-lysine unit is linked to the α-carboxyl group of the other to form a peptide bond, leaving the α-amino group as a side chain. ε-PL is water soluble, biodegradable, edible, and non-toxic toward humans and the environment, in addition to having broad-spectrum antibacterial activity. Thus, ε-PL and its derivatives have been of great interest for a broad range of industrial and biomedical applications such as food preservatives, emulsifying agent, dietary agent, biodegradable fibers, water absorbent hydrogels, drug-delivery carriers, and anticancer agent enhancer [1–3]. We have studied the molecular structure and the conformation of ε-PL in aqueous solution [6–8]. The pH-dependent IR, circular dichroism, and ^{1}H solution NMR spectra have indicated that ε-PL assumes a β-sheet conformation in basic aqueous solution and an electrostatically expanded conformation in acidic aqueous solution [6–8]. It is conceivable that the four methylene groups in the backbone of ε-PL endow the polymer with considerable conformational flexibility compared to the case of poly(α-L-lysine) (α-PL). We have also characterized the structure and the conformation of ε-PL and its derivatives in the solid-state by ^{13}C and ^{15}N solid-state NMR [9, 10]. These results indicated that ε-PL is a semicrystalline polymer with crystallinity of ca. 63% estimated by the measurements of ^{13}C spin–lattice relaxation time, T_1. A conformational model of ε-PL was also proposed in which the main chain takes a parallel β-sheet similar to the γ-form of nylon 6 [9]. Chemically modified derivatives of ε-PL, ε-PL/MO, and ε-PL/DC were prepared through reactions of ε-PL with methyl orange (MO) and dabsyl chloride (DC), respectively. In ε-PL/MO, side chain α-amino groups of ε-PL are involved in ionic bonds with methyl orange (MO) to form poly-ion complexes, (ε-PL)-$NH_3^+SO_3^-$-(MO). On the other hand, ε-PL is reacted with dabsyl chloride in ε-PL/DC to form covalent sulfonamide bonds, (ε-PL)-NH-SO_2-(DC). These chemically modified ε-PLs exhibit ^{15}N NMR signals characteristic of the binding mode at the α-amino groups [10].

Semi-transparent and water soluble films are formed by casting aqueous solution of ε-PL. In this work, we have prepared films cast from aqueous solution of ε-PL at various pHs. ^{13}C and ^{15}N CPMAS NMR spectra were measured and discussed in comparison with those of the ε-PL powder data. In order to confirm the carbamate formation, the measurements were done for films prepared under the degassed and the CO_2 bubbling conditions.

Experimental

Materials

Microbial ε-PL (free form, ε-PL) was kindly supplied by Chisso Corporation, Japan. The number-averaged molecular weight of ε-PL was determined to be 4,090, which corresponds to the degree of polymerization of 32 based on the unit molecular weight of 128. Ultra pure water, prepared by a Milli-QPlus ultra-pure water system (Millipore, USA) was used throughout the experiment. The degassed water was prepared by removing dissolved gas in water by a process of repeated freezing in liquid nitrogen, followed by pumping with rotary pump and then melting in a sealed flask. This process was repeated six times. Dry ice was used as a source of carbon dioxide gas. Dry ice was purchased from Uno Sanso, Japan. 1 M HCl aqueous solution used to adjust pH of aqueous solution of ε-PL was purchased from Wako Pure Chemicals Co., Japan.

Preparation of ε-PL film cast from aqueous solution

Cast films of ε-PL were prepared as follows unless otherwise noted. The ε-PL aqueous solution (3.5 wt%, 10 mL) was cast on a Teflon petri dish, dried in the air for 4 days, and subsequently dried in vacuum for 4 days at room temperature. The pH of ε-PL aqueous solution was adjusted by adding a small amount of 1 M HCl aqueous solution. The pH of ε-PL aqueous solution without pH adjustment (ε-PL cast film) was 9.3. The ε-PL film cast from degassed aqueous solution (CO_2 free ε-PL) was prepared as follows. The ε-PL solution dissolved in degassed water (2 wt%, 10 mL) was cast on a Teflon petri dish under CO_2 free environment and dried in vacuum for 2 days. ε-PL cast from aqueous solution with CO_2 bubbling (CO_2 bubbling ε-PL) was prepared as follows. CO_2 gas was bubbled gently through a stirred 2 wt% ε-PL aqueous solution in a glass vial for 6 h at room temperature. The pH of this solution was 6.3. This solution was cast on a Teflon petri dish and dried in vacuum for 2 days.

NMR measurements

^{13}C and ^{15}N CPMAS NMR spectra were measured with Chemagnetics CMX Infinity 300 operating at 75.6 and 30.0 MHz, respectively, at room temperature. The samples of a cast film cut into small pieces with scissors or in powder form were contained in a cylindrical rotor of zirconia ceramic. The rotor diameter was 5 mm, and the rotor was spun at 5.0 or 7.0 kHz. Contact time was 1 ms, and repetition time was 1 s. The number of accumulation was about 2,000 for ^{13}C and about 40,000 for ^{15}N. ^{13}C signal of methyl carbon of hexamethylbenzene was externally referenced to 17.35 ppm from tetramethylsilane. ^{15}N signal of glycine was externally referenced to 32.5 ppm from ammonia (liq. NH_3, 25 °C).

Results and discussion

Figure 1 shows the ^{13}C CPMAS NMR spectra of the ε-PL films cast from aqueous solution at various pHs along with the spectra of powder samples as a reference. Powder samples of ε-PL and ε-PL/HCl give well resolved ^{13}C peaks and the reported assignments are given in the figure [9]. In contrast, ε-PL films give less resolved spectra in the aliphatic carbon regions. As shown in Fig. 1e, the ε-PL film

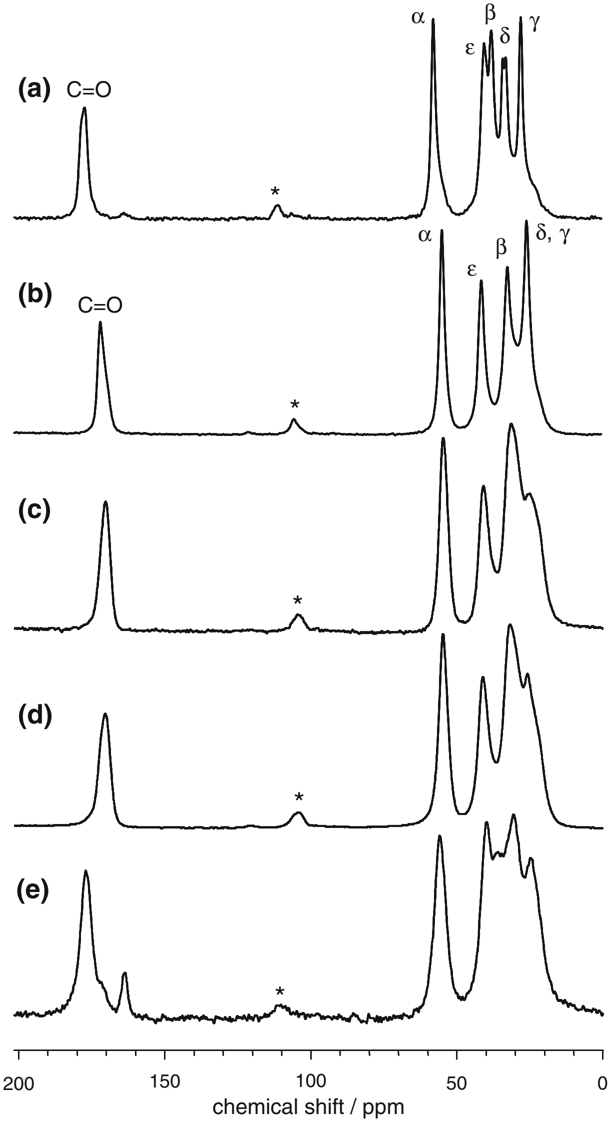

Fig. 1 ^{13}C CPMAS NMR spectra of (*a*) ε-PL powder, (*b*) ε-PL/HCl powder, and ε-PL film cast from aqueous solution of (*c*) pH 2.8, (*d*) pH 4.8, and (*e*) pH 9.3, respectively. A peak marked with an *asterisk* is a spinning side-band

cast from basic aqueous solution shows a small peak at 164 ppm with a shoulder at 171 ppm, in addition to a main peak at 177 ppm. The latter main peak corresponds to the C=O carbon peak of the ε-PL (Fig. 1a) [9]. The former peaks at 164 and 171 ppm are not observed in the spectra of the ε-PL films cast from acidic aqueous solution (Fig. 1c, d). These samples show a single peak at 171 ppm where C=O peak of ε-PL/HCL powder appears [10].

Table 1 ^{13}C chemical shifts of ε-PL and its derivatives in the solid state

Sample	ε-NHCO		α-NHCO	Cα
ε-PL[a]	178.6, 177.1			57.0
ε-PL/HCl[a]		171.1		54.9
ε-PL/BOC[a,b]		171.8		55.3
ε-PL/MO[a]		172.2		55.1
CO_2 free ε-PL	177.6			57.8
CO_2 bubbling ε-PL	176.5	170.9	163.7	55.7
ε-PL cast film	176.8	171.4	163.7	56.1

In ppm with respect to tetramethylsilane

[a] From Ref. [10]

[b] A quaternary carbon signal of BOC (*t*-butoxycarbonyl) appears at 79.4 ppm

Table 1 summarizes ^{13}C chemical shifts of NHCO carbons and Cα for ε-PL and its chemically modified derivatives, ε-PL/HCl, ε-PL/BOC (*t*-butoxycarbonyl), ε-PL/MO, CO_2 free ε-PL, ε-PL cast film, and CO_2 bubbling ε-PL. ε-PL/BOC is listed as a reference compound, which is a completely N_α-substituted ε-PL derivative [10]. ε-PL/MO (Methyl Orange) is also listed as a reference compound, which makes poly-ionic complex [10]. The pH-dependent IR, circular dichroism, and ^{1}H solution NMR spectra have indicated that ε-PL assumes a β-sheet conformation in basic aqueous solution and electrostatically expanded conformation in acidic aqueous solution [6–8]. We proposed a conformational model of crystalline component of ε-PL in which main chain takes the parallel β-sheet similar to the γ-form of nylon 6 and there exist intermolecular hydrogen bondings between amide C=O and NH groups [9]. We assume that ε-PL/HCl in the solid state takes a conformation similar to that in acidic solution without intermolecular hydrogen bondings. Thus, we attributed the downfield shift of ε-PL about 6 ppm compared with that of ε-PL/HCl to formation of hydrogen bondings.

Figure 2 compares ^{13}C CPMAS NMR spectra of three types of ε-PL films: (a) CO_2 free ε-PL, (b) ε-PL cast film, and (c) CO_2 bubbling ε-PL. ^{15}N CPMAS NMR spectra of the same samples are shown in Fig. 3. The CO_2 free ε-PL film prepared under CO_2 free environment shows only a single peak of C=O at 177.6 ppm. A small peak at 164 ppm in the spectrum of CO_2 free ε-PL (Fig. 2a) may originate in ε-PL powder sample supplied from the manufacturer [9]. In contrast, the CO_2 bubbling ε-PL and the ε-PL cast film show C=O peaks at 164 and 171 ppm, in addition to large C=O peak at 176.5 and 176.8 ppm, respectively. Primary amines are known to react with CO_2 to form alkylammonium alkylcarbamates according to a reaction shown in Scheme 2 [11–15].

^{13}C chemical shift of the carbonyl carbon of carbamates in solution falls in the 160–170 ppm range [11–13]. Thus, we attributed the peak observed at 164 ppm in the ε-PL cast film (Fig. 2b) and the CO_2 bubbling ε-PL film (Fig. 2c) to the carbonyl carbon of carbamates. The peak at 171 ppm can be attributed to the amide C=O carbon adjacent to the carbamated α-amino group. Bulky carbamate groups close to the amide C=O groups prevent formation of intermolecular hydrogen bonds and

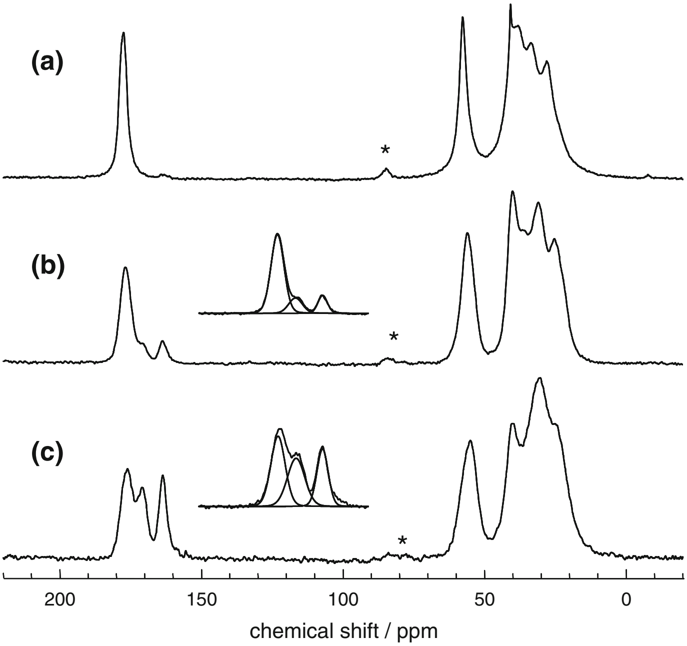

Fig. 2 ^{13}C CPMAS NMR spectra of ε-PL. (*a*) CO_2 free ε-PL, (*b*) ε-PL cast film, and (*c*) CO_2 bubbling ε-PL. A peak marked with an *asterisk* is a spinning side-band. Curve-fittings for C=O resonances are shown in the *insets*

destruction of hydrogen bondings caused upfield shifts. The curve fitting results (insets of Fig. 2) show that relative intensity ratio of peaks at 178, 171, and 164 ppm are 46, 27, and 27% for CO_2 bubbling ε-PL, and 78, 11, and 11% for ε-PL cast film, respectively. Thus, relative intensity ratio of peak at 178 and 171 ppm are 63 and 37% for CO_2 bubbling ε-PL, and 87 and 13% for ε-PL cast film. The present result indicates that 13% of amino groups of ε-PL forms carbamates when ε-PL aqueous solution (pH 9.3) was exposed to the air for 4 days. Table 2 summarizes ^{15}N chemical shifts of ε-PL, ε-PL/HCl, ε-PL/BOC, ε-PL/MO, CO_2 free ε-PL, CO_2 bubbling ε-PL, and ε-PL cast film. The most downfield side peaks are assigned to main chain amide nitrogen, ε-NHCO. ε-PL/BOC is a model compound of completely N_α-substituted ε-PL derivative and a peak at 92 ppm is assigned to side chain amide nitrogen, α-NHCO [10]. Therefore, we assigned peaks at about 92 ppm in Fig. 3b and c to side chain amide nitrogen of carbamates. We have measured ^{15}N spectra of various ε-PL derivatives and found that these chemically modified ε-PLs exhibit ^{15}N signals characteristic of the binding mode at the α-amino groups [10]. ^{15}N chemical shifts of CO_2 free ε-PL (Fig. 3a) are the same as those of ε-PL powder. This fact implies that side chain α-amino groups are not protonated. In contrast spectra of CO_2 bubbling ε-PL and ε-PL cast film show broad peak at about 34 ppm. We reported that side chain α-amino groups of ε-PL in ε-PL/MO are involved in ionic bonds with MO to form poly-ionic complexes [10]. The curve fitting results for α-amino groups of CO_2 bubbling ε-PL and ε-PL cast film (insets of Fig. 3) show

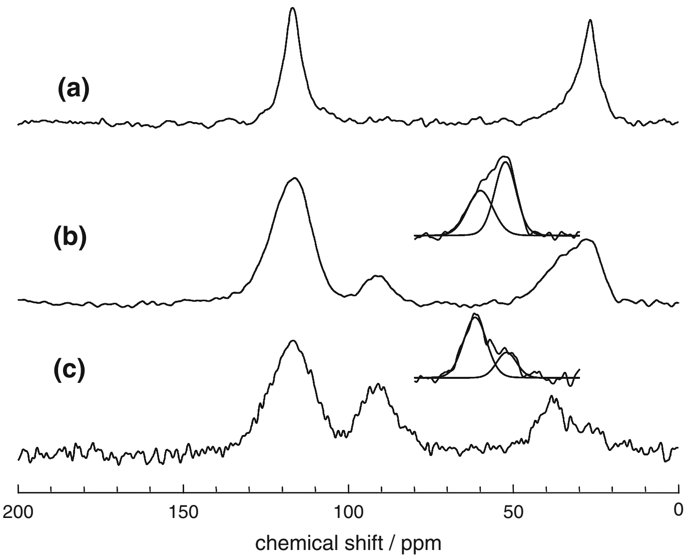

Fig. 3 ^{15}N CPMAS NMR spectra of ε-PL. (*a*) CO_2 free ε-PL, (*b*) ε-PL cast film, and (*c*) CO_2 bubbling ε-PL. Curve-fittings for amino groups are shown in the *insets*

$$CO_2 + 2RNH_2 \leftrightharpoons [RNH_3^+][RNHCOO^-]$$

Scheme 2 Reaction of primary amine and CO_2 to form carbamates

that the peaks are consisted of two peaks at about 27 and about 34 ppm. We attributed the former peak to nonprotonated amino groups and the latter to protonated amino groups which are involved in ionic bonds with carbamate anion to form poly-ionic complexes. A similar formation of carbamates was recently observed for α-PL [16].

Bubbling CO_2 through the stirred ε-PL aqueous solution resulted in noticeable increase in viscosity. Self-assembly of ionic pairs of ammonium groups and carbamate anions, $[RNH_3^+][RNHCOO^-]$, may lead to partially cross-linked polymer. This result shows that when we handle basic aqueous solution of ε-PL in the air, care has to be taken to avoid carbamate formation. Cross-linking methods of ε-PL by γ-ray irradiation [17] and with cross-linking reagents [3, 18] are reported. CO_2 bubbling method provides us with simple means of cross-linking of ε-PL.

Figure 2 shows a change of spectral pattern of aliphatic carbons of ε-PL as formation of carbamates proceeds. As we discussed in previous paper, CH_2 sequences in crystalline component of ε-PL have *trans*-zigzag conformation and show relatively sharp resonances. In contrast amorphous component has much broader resonances than the crystalline one. Furthermore, chemical shifts of the amorphous component are observed upfield compared with the crystalline one. The upfield shifts and the broadening of the CH_2 resonances are explained by the conformational heterogeneity and the γ-*gauche* effects in the amorphous components. However, crystalline component has β-sheet conformation with

Table 2 ^{15}N chemical shifts of ε-PL and its derivatives in the solid state

Sample	ε-NHCO	α-NHCO	α-NH_3^+		α-NH_2
			Free	Ion-complex	
ε-PL[a]	117				27
ε-PL/HCl[a]	123		44		
ε-PL/BOC[a]	120	92			
ε-PL/MO[a]	115			34	
CO_2 free ε-PL	118				27
CO_2 bubbling ε-PL	120	91		38[b]	27[b]
ε-PL cast film	116	92		34[b]	27[b]

In ppm with respect to liquid NH_3 at 25 °C

[a] From Ref. [10]

[b] Obtained from curve fitting

intermolecular hydrogen bondings as in ε-PL powder, since chemical shift of main carbonyl carbon peak is the same as in ε-PL powder regardless of the formation of carbamates.

Table 3 summarizes ^{13}C spin–lattice relaxation time in the laboratory frame, T_1, of C=O and Cα carbons measured by using Torchia's T1CP method [19]. The decay curves for C=O and Cα were fitted to a single exponential and biexponential function, respectively. Two components were observed for Cα: shorter T_1 is that of amorphous component and longer one corresponds to crystalline component. The degree of crystallinity was estimated from decay curve of Cα to be 56%.

Figure 4 shows a schematic diagram of structure of partially cross-linked ε-PL due to formation of carbamates. In order to make an ionic pair of ammonium groups and carbamate anions, the main chain CH_2 sequences must adopt twisted conformation, such as skewed *gauche* conformation. This conformational change may cause upfield shift due to γ-*gauche* effect and line broadening due to structural inhomogeneity. Characterization of ε-PL cast from aqueous solution and dynamics of formation of carbamates are currently studied and will be published in subsequent papers.

Table 3 ^{13}C spin–lattice relaxation time, T_1, of ε-PL cast from aqueous solution with CO_2 bubbling for 6 h

T_1/s	C=O			CH
	ε-NHCO 177 ppm	ε-NHCO 171 ppm	α-NHCO 164 ppm	Cα 56 ppm
Crystalline[a]	–	–	–	16
Amorphous[b]	24	18	23	1.7

Since resonances of Cβ–Cε are overlapping, T_1 of C=O and Cα were measured

[a] Since T_1 of C=O in crystalline component is very long, they were not measured

[b] Peak area of curve fitted each resonance was plotted against delay time

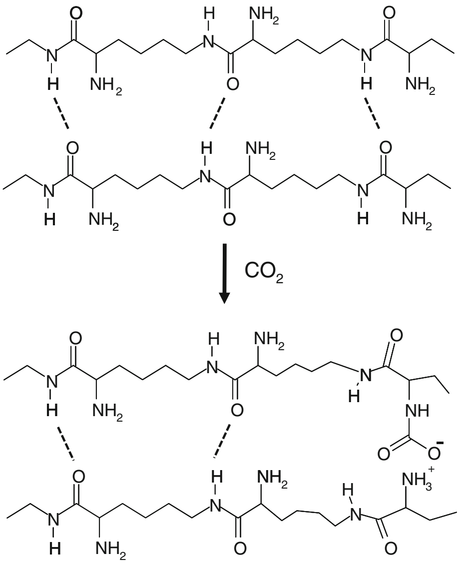

Fig. 4 Schematic diagram of structure of partially cross-linked ε-PL due to formation of carbamates. *Broken lines* show intermolecular hydrogen bondings

Conclusions

Formation of carbamates by amino groups of ε-PL and cross-linking of ε-PL was elucidated by using ^{13}C and ^{15}N solid-state NMR. It is a characteristic found in ε-PL cast from basic aqueous solution exposed to the air or gaseous CO_2. It is not observed in ε-PL cast from acidic aqueous solution and ε-PL cast from degassed aqueous solution under CO_2 free environment. The carbonyl carbon and amide nitrogen appear at 164 ppm in ^{13}C spectrum and 92 ppm in ^{15}N spectrum, respectively, due to formation of carbamates. In addition to these peaks a peak at 171 ppm appears. We attributed this peak to the amide C=O carbon adjacent to the carbamated α-amino group. Bulky carbamate groups close to the amide C=O groups prevent formation of intermolecular hydrogen bonds and destruction of hydrogen bondings caused upfield shifts. In order to make an ionic pairs of ammonium groups and carbamate anions, the main chain CH_2 sequences must adopt twisted conformation, such as skewed *gauche* conformation. This conformational change may cause upfield shift due to *γ-gauche* effect and line broadening due to structural inhomogeneity. These effects induce spectral pattern changes of aliphatic carbons as formation of carbamates proceeds. However, crystalline component remains *β*-sheet conformation with intermolecular hydrogen bondings as in ε-PL powder, since chemical shift of main carbonyl carbon peak do not changes regardless of the formation of carbamates. Bubbling CO_2 through the ε-PL aqueous solution resulted in noticeable increase in viscosity. Self-assembly of ionic pairs of ammonium groups and carbamate anions, $[RNH_3^+][RNHCOO^-]$, may lead to partial cross-linking of ε-PL. CO_2 bubbling method provides us with simple means of cross-linking of ε-PL. These results show that when we handle basic aqueous solution of ε-PL in the air, care has to be taken to avoid carbamate formation.

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