Structural analysis of microbial poly(ε-L-lysine)/poly(acrylic acid) complex by FT-IR, DSC, and solid-state 13C and 15N NMR

Shiro Maeda1, Yasuhiro Fujiwara1, Chizuru Sasaki2 and Ko-Ki Kunimoto3


Keywords: microbial polymer; poly(ε-L-lysine)/polymer complex; solid state NMR

INTRODUCTION

In recent years, there has been considerable interest in biopolymers due to the concern over the environmental impacts arising from the disposal of petroleum-based plastics. Poly(ε-L-lysine) (ε-PL) is one of the few poly(α-amino acid)s that are known to occur in nature.1,2 Microbial ε-PL is a product of a variant of Streptomyces albulus. In ε-PL molecules, the ε-amino group of an ε-L-lysine unit is linked to the γ-carboxyl group of the other unit to form a peptide bond, leaving the ε-amin group as a side chain. ε-PL is water soluble, biodegradable, edible and non-toxic to 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 agents, dietary agents, biodegradable fibers, water absorbent hydrogels, drug delivery carriers and anticancer agent enhancers.1 We have studied the molecular structure and the conformation of ε-PL in aqueous solutions.3–5 The pH-dependent infrared (IR), circular dichroism and 1H solution nuclear magnetic resonance (NMR) spectra have indicated that ε-PL assumes a β-sheet conformation in basic aqueous solutions and an electrostatically expanded conformation in acidic aqueous solutions.3–5 We have also characterized the structure and the conformation of ε-PL and its derivatives in the solid state by 13C and 15N solid-state NMR.6,7 These results indicated that ε-PL is a semi-crystalline polymer with a crystallinity of ~63%, as estimated by the measurements of 13C spin-lattice relaxation time in the laboratory frame. A conformational model of ε-PL was also proposed in which the main chain makes a parallel β-sheet similar to the γ-form of nylon 6.6 As tools for side chain functionalization, chemically modified derivatives of ε-PLs, ε-PL/methyl orange (MO) and ε-PL/dabsyl chloride (DC), were prepared through reactions of ε-PL with MO and DC. In ε-PL/MO, the side chain ε-amino groups of ε-PL are involved in ionic bonding with MO to form poly-ion complexes, (ε-PL)-NH3+···SO3-(MO). On the other hand, ε-PL reacts with DC in ε-PL/DC to form covalent sulfonamide bonds, (ε-PL)-NH-SO2-(DC). These chemically modified ε-PLs exhibit 15N NMR signals characteristic of the binding mode at the ε-amino groups.7 Polymer blends are widely used as a means of tailoring and modifying the characteristics of polymeric materials for various industrial and biomedical applications.8 Recently, a few studies on ε-PL-based polymer blends have been reported.9,10 In this work, a structural analysis of ε-PL polymer complexes with poly(acrylic acid) is carried out using 13C and 15N solid-state NMR, Fourier transform infrared spectroscopy and differential scanning calorimetry (DSC) measurements.

EXPERIMENTAL PROCEDURE

Materials

Microbial ε-PL (free form) was kindly supplied to us by Chisso Corporation (Tokyo, Japan) and was produced according to the reported procedure.5 The number-averaged molecular weight of ε-PL was determined to be 4090, which corresponds to a degree of polymerization of 32 based on a unit molecular weight of 128. The HCl salt form of ε-PL, ε-PL/HCl, was prepared with hydrochloric acid according to a reported procedure.6 Poly(acrylic acid) (PAA) with an average molecular weight of 5000 and other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used without further purification.

Sample preparation

ε-PL and PAA were dissolved separately in methanol (5 wt%). The ε-PL and PAA solutions were mixed with different compositions of ε-PL/PAA: (a) 1/3 (1/5.3), (b) 1/1 (1/1.8) and (c) 3/1 (1.7/1) in weight ratios (in unit molar ratios). As soon as the solutions were mixed, white precipitates appeared. After standing for ~2 h, the mixture was centrifuged. Precipitates were collected and washed with methanol and dried in vacuum for 3 days at ambient temperature. The three complex precipitates obtained are denoted hereafter as complex 1/3, complex 1/1 and complex 3/1.

1Division of Applied Chemistry and Biotechnology, Graduate School of Engineering, University of Fukui, Fukui, Japan; 2Department of Life System, Institute of Technology and Science, The University of Tokushima, Tokushima, Japan and 3Division of Material Engineering, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa, Japan.

Correspondence: Professor S Maeda, Division of Applied Chemistry and Biotechnology, Graduate School of Engineering, University of Fukui, Fukui, Fukui 910-8507, Japan.

E-mail: smaeda@u-fukui.ac.jp

Received 16 June 2011; revised 18 August 2011; accepted 1 September 2011; published online 19 October 2011
NMR measurements

$^{13}$C and $^{15}$N cross polarization and magic angle spinning (CPMAS) NMR spectra were measured with a Chemagnetics CMX Infinity 300 solid-state NMR spectrometer (Fort Collins, CO, USA) operating at 75.6 and 30.0 MHz, respectively, at room temperature. Samples were contained in a cylindrical rotor made of a zirconia ceramic. The rotor diameter was 5 mm and was spun at ~7.0 kHz. The contact time was 1 ms and the repetition time was 2 s. The $^{13}$C signal from the methyl carbon of hexamethylybenzene was externally referenced to 27.35 parts per million (p.p.m.) from tetramethylsilane. The $^{15}$N signal of glycine was externally referenced to 32.5 p.p.m. from ammonia (liquid NH$_3$).

The contact time was 1 ms and the repetition time was 2 s. The $^{13}$C signal from the methyl carbon of hexamethylybenzene was externally referenced to 27.35 parts per million (p.p.m.) from tetramethylsilane. The $^{15}$N signal of glycine was externally referenced to 32.5 p.p.m. from ammonia (liquid NH$_3$, 25°C).

IR and DSC measurements

The IR spectra were recorded on a JASCO FT/IR-620 spectrometer (JASCO, Hachioji, Japan) using KBr pellets. DSC was measured on a SII Nano Technology DSC6200 (SII Nano Technology, Chiba, Japan) using KBr pellets. DSC was measured on a SII Nano Technology DSC6200 (SII Nano Technology, Chiba, Japan) using KBr pellets. IR and DSC measurements

RESULTS AND DISCUSSION

Figure 1 shows DSC curves of pure ε-PL, ε-PL/PAA 1/1 complex and pure PAA. Both pure PAA and pure ε-PL show a major endothermic peak at their respective melting temperatures of 452 K and 445 K. Asano et al. reported a similar DSC profile for pure ε-PL. The melting points of the ε-PL/PAA complexes were observed at 508 K, 496 K and 510 K for complex 1/3, complex 1/1 and complex 3/1, respectively. The higher melting temperatures of the ε-PL/PAA complexes compared with both ε-PL and PAA imply the existence of strong intermolecular interactions between the ε-PL and PAA.

Figure 2 shows the IR spectra of pure PAA, ε-PL: PAA complexes cast from methanol solution, pure ε-PL/HCl and pure ε-PL powder in the spectral region of 1300–1800 cm$^{-1}$. For the pure PAA, two C=O stretching bands were observed at 1710 and 1730 cm$^{-1}$. For the pure ε-PL, ε-PL/HCl and pure ε-PL powder, the amide I band of ε-PL was in excess over that of PAA, the amide I band of ε-PL appeared at 1674 cm$^{-1}$ increased in intensity. In the spectrum of the ε-PL/PAA 3/1 complex, in which a unit molar content of ε-PL was in excess over that of PAA, the amide I band of ε-PL also appeared at 1647 cm$^{-1}$, and that for ε-PL/HCl appeared at 1670 cm$^{-1}$.

Thus, we concluded that for the ε-PL/PAA 1/3 and 1/1, ɛ-amino groups in the ε-PL component take the protonated cationic form $–\text{NH}_3^+$. In contrast to ε-PL/PAA 3/1, ɛ-amino groups in the ε-PL component take both the non-protonated form, $–\text{NH}_2$, and the protonated cationic form, $–\text{NH}_3^+$.

In Figure 3, the $^{13}$C CPMAS NMR spectra of pure ε-PL, ε-PL/PAA complexes and pure PAA are shown. Assignments for ε-PL$^+$ and PAA are shown in the Figures 3a and e, respectively. Peaks at ~180 p.p.m. in the ε-PL/PAA complexes were assigned to the carboxyl and carbonyl carbons of both polymers. A peak from the carboxyl carbon of the pure PAA was deconvoluted into two peaks as shown in the inset of Figure 3a. The downfield side peak at 183 p.p.m. corresponds to the carboxyl carbons of the dimeric form, and the upper field peak at 180 p.p.m. is due to the carboxyl carbons of the free form. Miyoshi et al. measured the $^{13}$C CPMAS NMR spectra of PAA/poly(ethylene oxide) complexes and concluded that three hydrogen bonding forms exist for the carboxyl group of PAA, namely (1) the complex form, with interpolymer hydrogen bonding between poly(ethylene oxide) molecules; (2) the dimeric form, with intrapolymer hydrogen bonding forms of carboxyl groups exist in the ε-PL/PAA complex, namely (1) the ionized form, (2) the dimeric form and (3) the free form. For the pure ε-PL and ε-PL/HCl, the amide I bands were observed at 1640 cm$^{-1}$ and 1672 cm$^{-1}$, respectively. Thus, these amide I frequencies could be used as marker bands for the $–\text{CH(\text{NH}_2})\text{-C(=O})$ and the $–\text{CH(\text{NH}_3^+})\text{-C(=O})$ moieties. Comparing the spectrum of the 1/3 complex to that of the 3/1 complex, the C=O band of the PAA component at 1710 cm$^{-1}$ reduced in intensity, and the amide I peak of the ε-PL component at 1674 cm$^{-1}$ increased in intensity. In the spectrum of the ε-PL/PAA 3/1 complex, in which a unit molar content of ε-PL was in excess over that of PAA, the amide I band of ε-PL also appeared at 1647 cm$^{-1}$, and that for ε-PL/HCl appeared at 1670 cm$^{-1}$.

Thus, we concluded that for the ε-PL/PAA 1/3 and 1/1, ɛ-amino groups in the ε-PL component take the protonated cationic form $–\text{NH}_3^+$. In contrast to ε-PL/PAA 3/1, ɛ-amino groups in the ε-PL component take both the non-protonated form, $–\text{NH}_2$, and the protonated cationic form, $–\text{NH}_3^+$.

In Figure 3, the $^{13}$C CPMAS NMR spectra of pure ε-PL, ε-PL/PAA complexes and pure PAA are shown. Assignments for ε-PL$^+$ and PAA are shown in the Figures 3a and e, respectively. Peaks at ~180 p.p.m. in the ε-PL/PAA complexes were assigned to the carboxyl and carbonyl carbons of both polymers. A peak from the carboxyl carbon of the pure PAA was deconvoluted into two peaks as shown in the inset of Figure 3a. The downfield side peak at 183 p.p.m. corresponds to the carboxyl carbons of the dimeric form, and the upper field peak at 180 p.p.m. is due to the carboxyl carbons of the free form. Miyoshi et al. measured the $^{13}$C CPMAS NMR spectra of PAA/poly(ethylene oxide) complexes and concluded that three hydrogen bonding forms exist for the carboxyl group of PAA, namely (1) the complex form, with interpolymer hydrogen bonding between poly(ethylene oxide) molecules; (2) the dimeric form, with intrapolymer hydrogen bonding
among PAA molecules; and (3) the free form, with no particular form of hydrogen bonding. Because IR measurements suggest that \( \varepsilon \)-amino groups in the \( \varepsilon \)-PL component take a protonated cationic form, \(-NH_3^+\), peaks at \( \sim 171 \) and \( 55 \) p.p.m. in the \( \varepsilon \)-PL/PAA complexes were assigned to the carbonyl carbons of the \( \varepsilon \)-PL component, in which the \( \varepsilon \)-amino groups were protonated and the \( \varepsilon \) carbons were adjacent to the \(-NH_3^+\) groups, respectively. As the content of PAA in the \( \varepsilon \)-PL/PAA complexes decreased, chemical shifts of the top of the peak of the downfield side peak, which were assigned to the carboxyl carbons of PAA, showed a downfield shift. Chemical shift values for carboxyl carbons of the ionized carbonyl group (\(-COO^−\)) in poly(sodium acrylate) have been reported at \( \sim 185 \) p.p.m.\(^{14}\) Because IR measurements suggest the existence of ionized carboxyl groups, we assigned the peak at 184 p.p.m. to the ionized carboxyl carbons. For the \( \varepsilon \)-PL/PAA 3/1 complex, the existence of two \( \varepsilon \)-PL components with \( \varepsilon \)-amino groups, \(-NH_2\), and with protonated \( \varepsilon \)-amino groups, \(-NH_3^+\), was also suggested. Thus, a peak at 178 p.p.m. was assigned to the carboxyl carbon of the pure \( \varepsilon \)-PL component in the complex. Therefore, the carboxyl and carboxyl carbon peaks at \( \sim 180 \) p.p.m. were curve fitted with a component of (1) the carboxyl carbon of the ionized carboxyl group, \(-COO^−\) (ionized form), at 184 p.p.m., (2) the carboxyl carbon that forms intrapolymer hydrogen bonds among PAA molecules (dimeric form), at 183 p.p.m. and (3) the carboxyl carbon that forms no particular form of hydrogen bonding (free form), at 180 p.p.m., and the two \( \varepsilon \)-PL components mentioned above, at 171 and 178 p.p.m.\(^{14}\) The results from curve fitting are shown in the insets of Figures 3b–d.

Figure 3 shows a significant change in the spectral pattern of the aliphatic carbons of the \( \varepsilon \)-PL component in \( \varepsilon \)-PL/PAA complexes, as compared with that of pure \( \varepsilon \)-PL. As we discussed in our previous paper, the \( \text{CH}_2 \) sequences in the crystalline component of \( \varepsilon \)-PL have a trans-zigzag conformation and show relatively sharp resonances. In contrast, the amorphous component has much broader resonances than the crystalline component. Furthermore, chemical shifts of the amorphous component were observed upfield compared with the crystalline component. The upfield shifts and the broadening of the \( \text{CH}_2 \) resonances in \( \varepsilon \)-PL/PAA complexes are explained by the conformational heterogeneity and the \( \gamma \)-gauche effects in the amorphous components. Moreover, because \( \varepsilon \)-PL/PAA complexes are ionic compounds, the ionic environment also induced chemical shift changes.

Figure 4 shows the \( ^{15}\text{N} \) CPMAS NMR spectra of \( \varepsilon \)-PL, \( \varepsilon \)-PL/PAA 3/1, \( \varepsilon \)-PL/PAA 1/1, \( \varepsilon \)-PL derivative with MO and HCl, \( \varepsilon \)-PL/PAA (3/1) complex and \( \varepsilon \)-PL/PAA (1/3) complex and \( \varepsilon \)-PL/PAA 3/1 complex.

Table 1 \( ^{15}\text{N} \) NMR chemical shifts of \( \varepsilon \)-PL and its derivatives (p.p.m. from liquid \( \text{NH}_3 \) (25 C))

<table>
<thead>
<tr>
<th>( \varepsilon )-PL and its derivatives</th>
<th>( \text{NH}_3^+ )</th>
<th>( \text{NH}_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \varepsilon )-PL(^a)</td>
<td>116.9</td>
<td>26.6</td>
</tr>
<tr>
<td>( \varepsilon )-PL/HCl(^a)</td>
<td>122.7</td>
<td>44.0</td>
</tr>
<tr>
<td>( \varepsilon )-PL/MO(^a)</td>
<td>116.1</td>
<td>34.1</td>
</tr>
<tr>
<td>( \varepsilon )-PL/PAA( 1/3)</td>
<td>119.6</td>
<td>38.1</td>
</tr>
<tr>
<td>( \varepsilon )-PL/PAA (1/1)</td>
<td>121.5</td>
<td>38.8</td>
</tr>
<tr>
<td>( \varepsilon )-PL/PAA (3/1)</td>
<td>119.8</td>
<td>37.0</td>
</tr>
<tr>
<td>Chitosan(^b)</td>
<td></td>
<td>26.3</td>
</tr>
<tr>
<td>CS/PAA(^b)</td>
<td></td>
<td>21.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>31.6</td>
</tr>
</tbody>
</table>

Abbreviations: CS, chitosan; \( \varepsilon \)-PL, poly(\( \varepsilon \)-lysine); MO, methyl orange; NMR, nuclear magnetic resonance; PAA, poly(acrylic acid); p.p.m., parts per million.

\(^a\)Taken from reference Maeda et al.\(^{15}\)

\(^b\)Taken from reference Sakurai et al.\(^{15}\)
that these chemically modified ε-PLs exhibit 15N signals that are characteristic of the binding mode at the α-amino groups. We concluded that the side chain α-amino groups of ε-PL in ε-PL/MO are involved in ionic bonding with MO to form poly-ionic complexes. The ε-PL/PAA 1/1 complex shows a broad peak at ~38 ppm. We attribute this peak to protonated amino groups that are involved in ionic bonding with ionized carboxylic groups in PAA to form the poly-ionic complex ε-PL-NH₃⁺...OOC-PAA. In the spectrum of ε-PL/PAA 3/1 the peak of the non-protonated α-amino groups, −NH₂, also appeared at 26.3 ppm, and that for the protonated α-amino groups, −NH₃⁺, appeared at 37.0 ppm. The spectrum of the ε-PL/PAA 1/3 complex is similar to that of ε-PL/PAA 1/1 (data not shown). These results are consistent with the DSC and IR results. Measurements of the spin-lattice relaxation time in both the laboratory and rotating frames, and investigation of the morphology and chain dynamics of the ε-PL/PAA complex will be performed and published in subsequent papers.

CONCLUSION

The DSC measurements showed that the ε-PL/PAA 1/1 complex has a higher melting temperature than both pure ε-PL and pure PAA, implying the formation of strong intermolecular interactions between ε-PL and PAA. The IR measurements suggested the existence of protonated amino groups, −NH₃⁺, in the ε-PL component and ionized carboxyl groups, −COO⁻, in the PAA component of the ε-PL/PAA complexes. In the 13C NMR spectra of the ε-PL/PAA complexes, the carboxyl carbon of PAA is resolved into three components, namely (1) the ionized form, or the interpolymer ion-complex between ε-PL and PAA; (2) the dimeric form, or the intrapolymer hydrogen bonding among PAA molecules; and (3) the free form, with no particular form of hydrogen bonding. These results, along with the 15N NMR data confirm the formation of poly-ion complexes between ε-PL and PAA, as ε-PL-H₂N⁺...−OOC-PAA, in the ε-PL/PAA complexes.