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Structural investigation of microbial poly(ε-L-lysine) derivatives with azo dyes by solid-state ^{13}C and ^{15}N NMR

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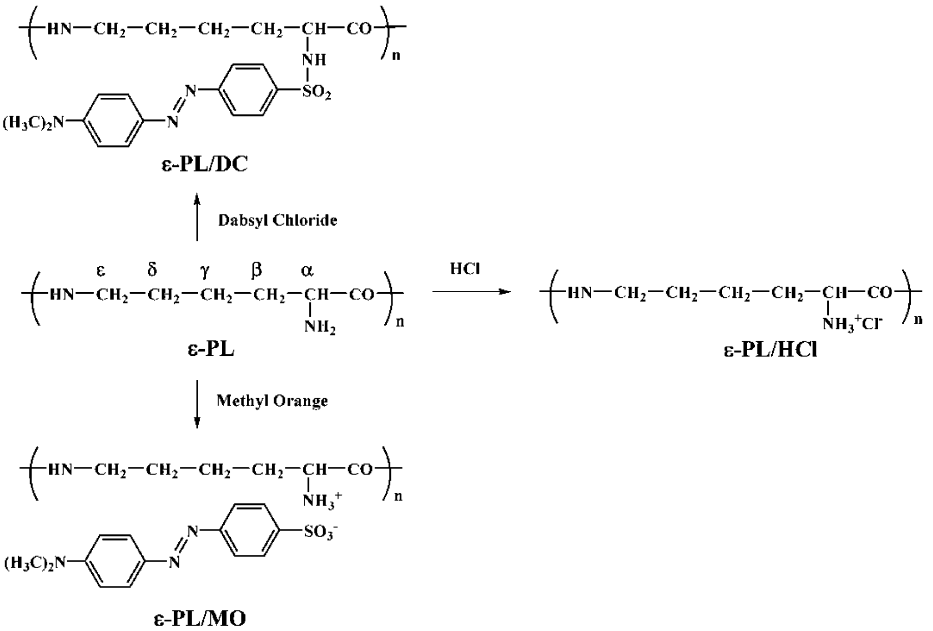
Summary

Solid-state high-resolution ^{13}C and ^{15}N NMR spectra of microbial poly(ε-lysine) (ε-PL) derivatives with azo dyes have been measured. Chemically modified derivatives of ε-PL, ε-PL/MO and ε-PL/DC were prepared through reactions with methyl orange (MO) and dabsyl chloride (DC), respectively. Side chain α-amino groups of ε-PL in ε-PL/MO are involved in ionic bonds with methyl orange to form poly-ion complexes, (ε-PL)-$NH_3^+SO_3^-$-(MO). On the other hand, ε-PL is allowed to react with dabsyl chloride in ε-PL/DC to form covalent sulfonamide bonds, (ε-PL)-NH-SO_2-(DC). These chemically modified ε-PL's exhibit ^{15}N NMR signals characteristic of the binding mode at the α-amino groups. The spectral analysis reveals that the ε-PL/DC sample contains a small amount of ion complexes with MO. It has been shown that ^{15}N solid-state NMR is a useful tool for structural determination of ε-PL and its derivatives.

Introduction

Poly(ε-L-lysine) (ε-PL) is one of a few poly(amino acid)s which are known to occur in nature [1]. Microbial production of ε-PL was first discovered by Shima and Sakai from a culture filtrate of *Streptomyces* and on physicochemical properties of the polymer were reported in some detail [2-5]. Due to antibacterial activities of microbial ε-PL against broad spectrum of microorganisms, ε-PL has been used as a preservative for various food products [6]. From a viewpoint of the molecular structure, ε-PL is regarded as an α-amino substituted derivative of nylon 6. However, ε-PL is water-soluble and biodegradable unlike nylon 6. We have studied the molecular structure and the conformational properties of ε-PL in aqueous solution [7-9]. IR and CD spectroscopic studies showed that ε-lysine oligomers form a β-sheet structure in aqueous solution, the content of which is dependent on the chain length and the pH. Recently, we have also investigated the solid structure of ε-PL by FT-IR, Raman



Scheme 1. Preparation of ε-PL derivatives.

and solid-state ^{13}C NMR spectra and demonstrated that ε-PL assumes a parallel β-sheet conformation similar to that of γ-type nylon 6 [10].

When methyl orange (MO, 4-dimethylaminoazobenzene-4'-sulfonic acid) is added to an aqueous solution of ε-PL (see scheme 1), the ε-PL/dye complex precipitates out of solution. This procedure has been generally utilized for determination of free amino groups in peptides and proteins [11]. In the reaction with an amino labeling reagent, dabsyl chloride (DC, 4-dimethylaminoazobenzene-4'-sulfonyl chloride), ε-PL also forms water-insoluble ε-PL/DC. Since polymers containing azobenzene moieties have potential application as polymeric dyes and optical materials etc, it is of importance to study the structural features of the ε-PL/azo dye complexes. ε-PL/Boc (*t*-butoxycarbonyl) is also prepared as a reference compound which is completely *N*-substituted ε-PL derivative. ^{13}C and ^{15}N solid-state high-resolution NMR measurements of ε-PL and its derivatives are reported and molecular interaction between ε-PL and the dye is discussed.

Experimental

Materials

Microbial ε-PL (free form, ε-PL) is a gift from Chisso Corporation produced according to the reported procedure [7]. 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. Methyl orange (MO), Boc anhydride (Boc_2O), dabsyl chloride (DC) and other reagents were purchased from Tokyo Kasei

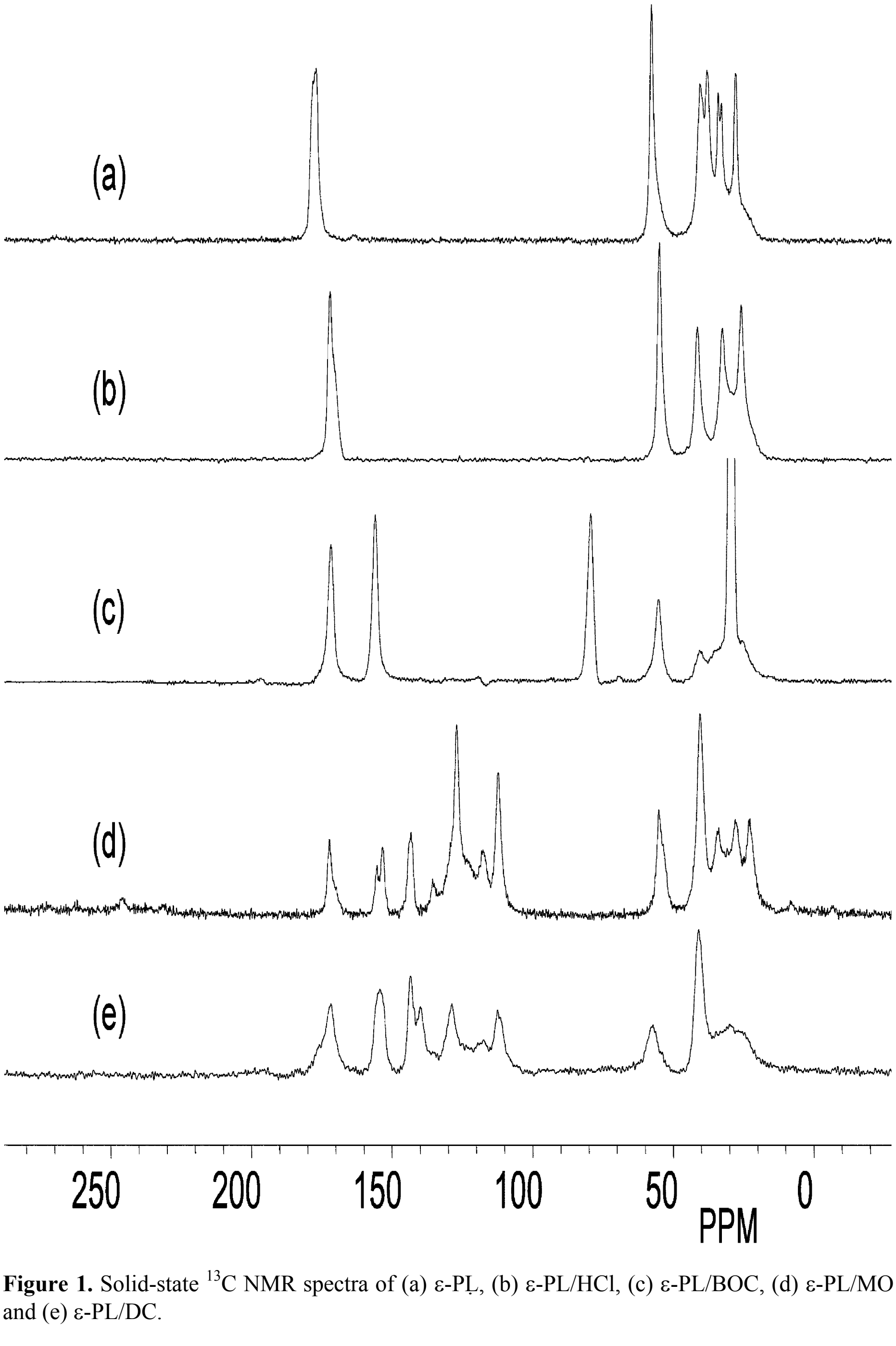


Figure 1. Solid-state ^{13}C NMR spectra of (a) ε-PL, (b) ε-PL/HCl, (c) ε-PL/BOC, (d) ε-PL/MO and (e) ε-PL/DC.

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

sample	ε-PL						dye	
	ε-NHCO	Cα	Cβ	Cγ	Cδ	Cε	α-NHCO	-N(CH$_3$)$_2$
ε-PL[b]	178.6,177.1	57.0	36.7	25.0	30.9	41.6		
ε-PL/HCl	171.1	54.9	32.5	25.8	25.8	41.4		
ε-PL/BOC[c]	171.8	55.3	-	25.4	25.4	40.6	156.1	
ε-PL/MO[d]	172.2	55.1	33.8	23.1	28.0	40.5		40.5
ε-PL/DC[d]	171.9	57.7	20 - 40			40.5		40.5

a) In ppm with respect to tetramethylsilane.
b) From reference [10].
c) A quarternaly carbon signal of Boc appears at 79.4 ppm.
d) Aromatic carbon peaks of azo dyes appear in the region from 112 to 155 ppm.

Kogyo, Japan, and used without further purification. The HCl salt form of ε-PL, ε-PL/HCl was prepared with hydrochloric acid according to the reported procedure [8]. ε-PL/MO was prepared as follows. ε-PL (26 mg, 67 μmol) and MO (65.4 mg, 2 mmol) were mixed and dissolved in 2000 ml of 0.2 M phosphate buffer solution (pH 6.0) and the pH was adjusted to 6.0-7.0 by adding 0.1-1.0 M hydrochloric acid. The molar ratio of [MO]/[lysyl residue] used here corresponds to 1.0. The solution was allowed to react for 4 h at room temperature. When the solution volume was reduced to about 150 ml by evaporation, a precipitate was formed. After centrifugation for 15 min, the orange products were taken out and dried in vacuo. ε-PL/DC was prepared according to a conventional Schotten-Baumann reaction. To a 100 ml MeOH solution of dabsyl chloride (162 mg, 0.5 mmol) containing triethylamine (152 mg, 1.5 mmol), 64 mg of ε-PL (0.5 mmol) was added. The mixture was allowed to stand at room temperature for 20 h. The resulted reddish-orange precipitate was collected by centrifugation. The product was washed with distilled water several times. ε-PL/Boc was prepared through *tert*-butoxycarbonylation using Boc anhydride. To a mixture of ε-PL (18 mmol) and triethylamine (49 mmol) in methanol, 18 mmol of Boc anhydride was added, and the mixture was continuously stirred for 3 h at refluxing temperature. The mixture was evaporated and re-dissolved in chloroform. By adding hexane, the crude product was obtained. The white product was washed with hexane several times.

NMR measurements

^{13}C and ^{15}N CPMAS NMR spectra were measured with Chemagnetics CMX Infinity 300 operating at 75.6 MHz and 30.0 MHz, respectively, at room temperature. The samples in powder form were contained in a cylindrical rotor of zirconia ceramic. The rotor diameter was 5mm for ^{13}C and 7.5 mm for ^{15}N, and the rotor was spun at 7.0 kHz and 5.0 kHz, respectively. Contact time was 1ms, and repetition time was 2 sec. The number of accumulation was about 12,000 for ^{13}C and about 40,000 for ^{15}N. The ^{13}C and ^{15}N chemical shifts were externally referenced to tetramethylsilane and liquid ammonia (NH$_3$, 25ºC), respectively.

Results and Discussion

The degree of chemical modification was evaluated from ^{1}H NMR spectra of DMSO-*d*$_6$ solutions, by using integral values obtained from the number of -CH$_2$- protons of ε-PL and appropriate protons of chemical modifiers. The apparent degree of chemical modification was 90 % for ε-PL/MO, 83 % for ε-PL/DC and more than 95 % for ε-PL/Boc.

Figure 1 shows the solid-state ^{13}C CPMAS NMR spectra of ε-PL and its derivatives. ^{13}C chemical shifts are summarized in Table 1, as well as assignments for ε-PL and ε-PL/HCl, and assignments for ε-PL derivatives. As previously reported, the peaks of ε-PL and ε-PL/HCL are well resolved and assigned to the individual carbons of the repeating unit [10, 12]. The C=O carbon signal of ε-PL/HCl shifts to upfield ca. 6 ppm compared to that of ε-PL. Signals of other chain carbons, except Cγ, also show upfield shift. The chemical shift displacements are interpreted mainly as the substituent effect of the NH_3^+ group upon protonation.

The spectrum of ε-PL/Boc shows side-chain α-NHCO signal at 156.1 ppm in addition to main-chain ε-NHCO signal at 171.8 ppm. Peak intensity of the former is almost the same as that of the latter, which implies that α-NH$_2$ groups of ε-PL are almost completely substituted with Boc to make amide bond, (ε-PL)-NH-CO-(Boc).A peak at 29.4 ppm which goes through the ceiling is assigned to three identical methyl carbons of *tert*-butoxy group. The signals of the ε-PL/Boc in the backbone -CH$_2$- region (20 – 40ppm) are somewhat less resolved. The broad -CH$_2$- peaks suggest the conformational inhomogeneity of the CH$_2$ chain due to the existence of Boc in the side chain of ε-PL/Boc.

The spectral pattern of ε-PL/MO in the aliphatic carbon region is similar to that of ε-PL/HCl. The Cε peak of ε-PL/MO is more intense compared to that of ε-PL and ε-PL/HCl. This is because this peak has contribution from the *N*-methyl carbon of MO molecule. The peaks in the 112-155 ppm region are due to the aromatic carbons of ε-PL/MO. Similarity of the ^{13}C chemical shifts between ε-PL/HCl and ε-PL/MO suggests the chemical shifts are mainly dominated by substituent effect of the NH_3^+.

The signals of the ε-PL/DC in the backbone -CH$_2$- region (20 – 40ppm) are much less resolved like ε-PL/Boc. The broad and unresolved -CH$_2$- peaks suggest the conformational inhomogeneity of the CH$_2$ chain due to the existence of bulky aromatic groups in the side chain of ε-PL/DC. The signals in the aromatic carbon region of both ε-PL/MO and ε-PL/DC are overlapping each other. Therefore, it is difficult to assign the peaks individually and to distinguish covalently bonded sulfonamide linkage from poly-ionic complex formation.

As mentioned above, Boc must completely make a covalent bond with α-amino group of ε-PL to make amide bond, (ε-PL)-NH-CO-(Boc). An amino labeling reagent DC is also expected to be covalently bonded to α-amino group of ε-PL to make sulfonamide bond, (ε-PL)-NH-SO$_2$-(DC), whereas MO is expected to be negatively ionized to form ionic bond, (ε-PL)-$NH_3^+SO_3^-$-(MO). The mechanism of the formation of ε-PL derivatives, however, is not clearly elucidated through ^{13}C NMR measurements. Therefore, we have carried out ^{15}N solid-state NMR measurements of these ε-PL derivatives in order to specify the interaction between α–amino moieties and azo dye or Boc groups.

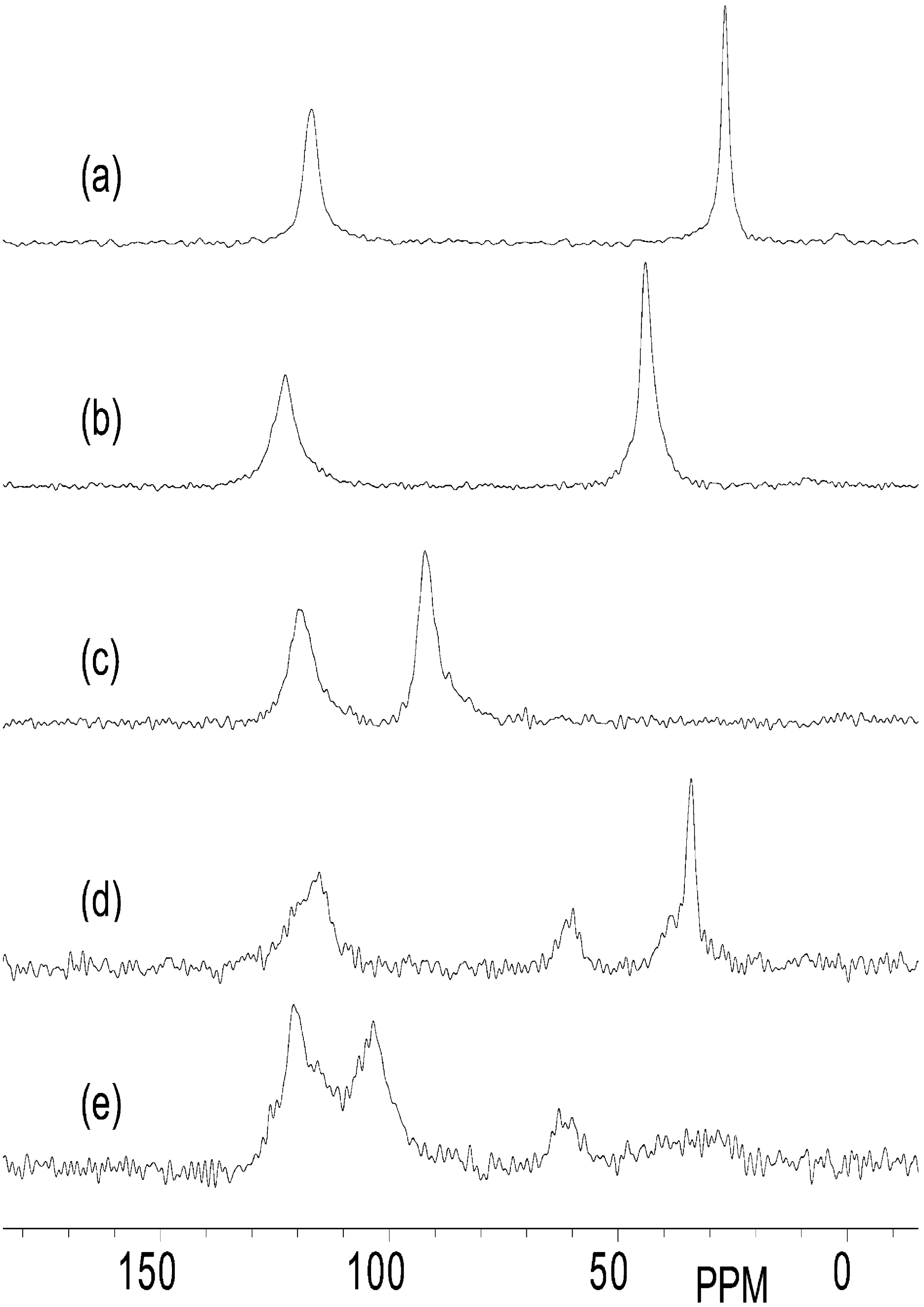


Figure 2. Solid-state ^{15}N NMR spectra of (a) ε-PL, (b) ε-PL/HCl, (c) ε-PL/BOC, (d) ε-PL/MO and (e) ε-PL/DC.

Figure 2 shows the ^{15}N CPMAS spectra of ε-PL and its derivatives [13]. The chemical shifts and their assignments are summarized in Table 2. ε-PL exhibits two distinct ^{15}N peaks at 117 and 26.6 ppm. The former and the latter peaks are assigned to the main chain NHCO and the α-NH_2 groups in comparison with nylon 6 [14], and chitosan and chitin [15-17]. In ε-PL/HCl, the NHCO and the α-NH_2 peaks are observed at 123 and 44 ppm, respectively. These downfield shifts are attributed to protonation of the α-amino group to form α-$NH_3^+Cl^-$ salt in ε-PL/HCl. Similar shift is observed in chitosan film when 2 % formic acid solution is used as casting solvent [15, 16].

ε-PL/Boc also exhibits two distinct ^{15}N peaks at 120 and 92.2 ppm. The former and the latter peaks are assigned to the main chain ε-NHCO and the side-chain α-NHCO in comparison with nylon 6 [14], and chitosan and chitin [15-17].A signal of α-amino group completely disappears. These findings suggest that Boc makes completely a covalent bond with α-amino group of ε-PL to form side chain amide linkage, which is in good accordance with the results of ^{13}C NMR measurements.

ε-PL/MO shows three ^{15}N peaks at 115, 34.1 and 59.9 ppm. The last signal is assigned to dimethyl amino nitrogen of the dye moiety [18, 19]. The first and the second one are assigned to the main chain CONH and the α-NH_3^+ group, respectively. The ^{15}N chemical shift of the α-NH_3^+ is between those of ε-PL and ε-PL/HCl. Since analogous chemical shift is reported for chitosan/poly(acrylic acid) [15, 16, 20], the formation of poly-ion complex between ε-PL and MO, (ε-PL)-α-NH_3^+ SO_3^--(MO), is clearly elucidated. Although the azo ^{15}N signals are expected around 510 ppm [18, 19], no such peaks are observed in this region. Because azo nitrogen nuclei are far from protons, ^1H-^{15}N cross polarization efficiency must be much weaker than the other protonated nitrogen nuclei and the signal of azo nitrogen nuclei does not appear in CPMAS spectra.

ε-PL/DC shows ^{15}N peaks at 121, 102, 61.6 ppm. The last peak is due to the dimethyl amino group. The first and the second peak are assigned to the main chain CONH and the sulfonamide groups, suggesting the formation of the covalent (ε-PL)- α- $NHSO_2$-(DC) bond.

Table 2. ^{15}N NMR chemical shifts of ε-PL and its derivatives in the solid-state[a)].

sample	ε-NHCO	α-$NHSO_2$	α-NHCO	α-NH_3^+		α-NH_2	-$N(CH_3)_2$
				free	ion-complex		
Nylon 6 [b)]	116.5						
ε-PL	117					26.6	
ε-PL/HCl	123			44.0			
ε-PL/BOC	120		92.2				
ε-PL/MO	115				34.1		59.9
ε-PL/DC	121, 113	102			32.1		61.6
Chitosan [c)]						21.2	
CS/PAA [d)]					31.6		

a) In ppm with respect to liquid NH_3 at 25°C.
b) α-crystalline form [14].
c) References [15-17].
d) Chitosan/poly(acrylic acid) [15, 16, 20].

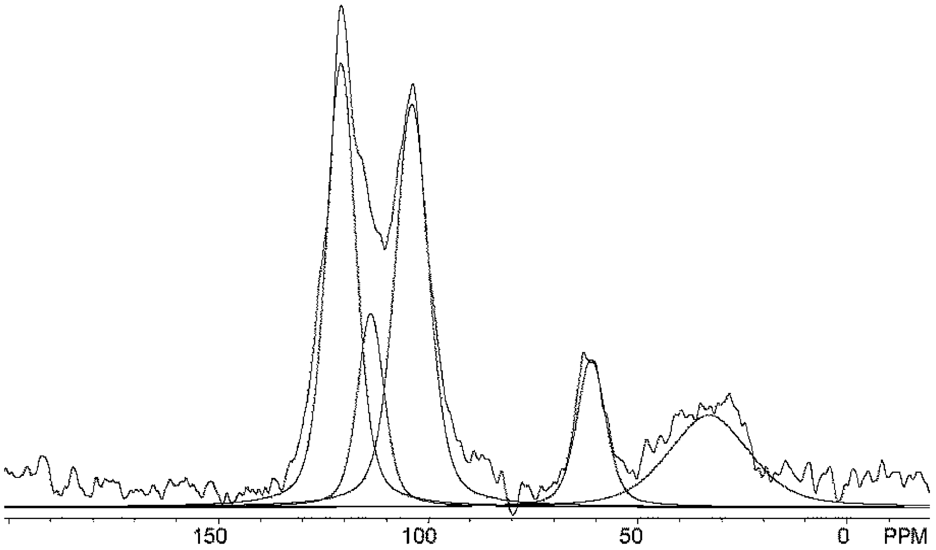


Figure 3. Curve fitting for ^{15}N NMR spectrum of ε-PL/DC.

There is also a weak peak at 32.1 ppm which coincides with the chemical shift of the α-NH_3^+ group. This finding suggests the existence of small amount of ε-PL/MO in ε-PL/DC. To make this point clear, the spectrum of ε-PL/DC is curve fitted as shown in Figure 3[21]. The curve fitting result shows a small peak at 113 ppm which can be assigned to a signal of main chain amide group, ε-$\underline{N}$HCO-CH(α-NH_3^+). By estimating the relative peak area of the 121 and 113 ppm peaks, we conclude that the ε-PL/DC sample contains a small amount of ion complexes with MO.

Conclusions

The ε-PL derivatives with azo dyes were investigated by using solid-state ^{13}C and ^{15}N NMR. It has been shown that side chain α-amino group of ε-PL does not make a covalent bond with methyl orange (MO) but forms poly-ion complex, (ε-PL)-$NH_3^+SO_3^-$-(MO). On the other hand, dabsyl chloride (DC) makes covalent bond with ε-PL to form sulfonamide, (ε-PL)-NH-SO_2-(DC). However, a few tens percent of DC change to MO by hydrolysis to form poly-ion complex, (ε-PL)-$NH_3^+SO_3^-$-(MO). It has been shown that ^{15}N solid-state NMR is useful tool for structural determination of ε-PL and its derivatives.

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