Poly(ethylene oxide-*co*-*β*-benzyl L-aspartate) Block Copolymers: Influence of the Poly(ethylene oxide) Block on the Conformation of the Poly(*β*-benzyl L-aspartate) Segment in Organic Solvents

S. Cammas,[†,‡] A. Harada,[‡,§] Y. Nagasaki,[‡,§] and K. Kataoka*[,‡,§]

Institute of Biomedical Engineering, Tokyo Women's Medical College, 8-1 Kawada, Shinjuku-ku, Tokyo 162, Japan, International Center for Biomaterials Science, Research Institute for Biosciences, Science University of Tokyo, Yamazaki 2669, Noda 278, Japan, and Department of Materials Science and Technology, Science University of Tokyo, Yamazaki 2641, Noda 278, Japan

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ABSTRACT: The conformation of the poly(*β*-benzyl L-aspartate) segment in the poly(ethylene oxide-*co*-*β*-benzyl L-aspartate) (PEO/PBLA) block copolymers (M_w of PEO = 5000 and 20 units of *β*-benzyl L-aspartate) was investigated by ^{1}H NMR, by specific optical rotation measurements, and by 2D ^{1}H,^{1}H NOESY NMR in chloroform, dimethyl sulfoxide (DMSO), and mixtures of chloroform/DMSO. The comparison between the ^{1}H NMR spectra of the block copolymer in $CDCl_3$ and the one in DMSO-d_6 showed that the PBLA blocks adopt a different conformation depending upon the solvents. The specific rotation of the block copolymer at 546 nm demonstrated that the PBLA segments adopt a left-handed α-helix conformation in chloroform. PBLA homopolymer with the same molecular weight as the PBLA segment in the block copolymer showed no evidence of α-helix formation in the same conditions, indicating that the PEO segment in the block copolymer is essential to allow PBLA to take the α-helix structure. On the other hand, the specific rotation of the PEO/PBLA block copolymer in DMSO at the same wavelength showed that the PBLA segments have a random-coil conformation in this solvent. Moreover, the measurement of the specific rotation of the block copolymer in mixtures of x% $CHCl_3$/(100 − x)% DMSO ($0 \le x \le 100$) demonstrated that the left-handed α-helix conformation adopted by the PBLA segments in chloroform is stable. Conversely, the PBLA homopolymer cannot be solubilized in mixtures of $CHCl_3$/DMSO, although this homopolymer is soluble in pure $CHCl_3$ and in pure DMSO. The 2D ^{1}H,^{1}H NOESY NMR spectrum in $CDCl_3$ gave evidence of interactions between the methylene protons ($CO_2CH_2C_6H_5$) of the PBLA segments and the methylene protons of the PEO blocks on one hand and between the benzyl protons of the PBLA segments and the methylene protons of the PEO blocks on the other. Such interactions are not observed in DMSO-d_6. Thus, the stability of the left-handed α-helix conformation of the PBLA blocks (having low molecular weights) in chloroform can be explained by the interactions existing between the PBLA and the PEO blocks. Moreover, these interactions also allow the solubilization of the block copolymer in mixtures of $CHCl_3$/DMSO.

Introduction

Recently, polymeric micelles prepared from poly(ethylene oxide-*co*-*β*-benzyl L-aspartate) (PEO/PBLA) block copolymers and their derivatives have received much interest as potentially powerful drug carriers.[1,2] Indeed, it has been shown that such polymeric micelles that are stable in aqueous medium can solubilize hydrophobic drugs in their inner core.[1−5] The PEO/PBLA polymeric micelles are obtained by dialysis against water of an organic solution of the block copolymers; therefore, the outer shell of the carrier is made of hydrophilic PEO chains, whereas the inner core is made up by hydrophobic poly(amino acid benzyl ester), PBLA.[6,7] Their physico-chemical properties (cmc, diameter, drug loading capacity, etc.)[1,2,6−8] and their biological characteristics (cytotoxicity, anticancer activity, etc.)[3−5,9,10] have been extensively studied. However, fewer studies have been done to characterize the conformation of the PBLA blocks in organic solvents. Since the micelles are formed from an organic solution of the block copolymers and some properties of the carriers (stability, drug loading capacity, etc.) might depend on the conformation of the PBLA blocks, the knowledge of the conformation of the hydrophobic segments in the PEO/PBLA copolymers in organic solvents appears to be of importance.

In addition, it is well-known that polypeptides show different conformations in solution and in solid state: α-helix, *β*-sheet, and random coil. The conformation adopted by the polypeptides depends on the side chain, the solvent, the temperature, and the pH.[11−15] Furthermore, transitions between the different conformations can be observed as a function of the temperature and the pH, as well as by changing the nature and the concentration of the solvent.[11−15] Also, the formation of a stable helical structure was shown to depend on the molecular weight of the poly(amino acid).[11−16] Zimm and Bragg had observed a critical molecular weight to obtain a stable α-helix conformation as well as to induce a helix−coil transition.[17]

One approach to stabilize the α-helix of a poly(amino acid) is to copolymerize it with another poly(amino acid) or with a different polymer. This type of copolymer can be used for the conformational study of more complex molecules such as proteins. Teramoto et al. have studied the conformational induction in block[18−20] and in sequential[21−24] copolypeptides of *γ*-benzyl L-glutamate and *ϵ*-carbobenzoxy-L-lysine or -L-alanine in different solvents (*m*-cresol, dichloroacetic acid, etc.). They concluded that the helical conformation of the copolypeptide is stabilized by a conformational induction operative between adjacent residues of a sequential copolypeptide[22] and by an interaction from the flanking *γ*-benzyl L-glutamate blocks for the block copolymer.[18] In the

* To whom correspondence should be addressed.
† Tokyo Women's Medical College. Tel: +81 3-3353-8111 (ext. 30232).
‡ Research Institute for Biosciences, Science University of Tokyo. Tel: +81 471-23-9771.
§ Department of Materials Science and Technology, Science University of Tokyo. Tel: +81 471-24-1501 (ext. 4310).
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latter case, the central ϵ-carbobenzoxy-L-lysine block is forced to take a helical conformation.[18] However, no evidence of intermolecular and/or intramolecular interactions was reported to explain the stabilization of the helical conformation of the poly(amino acid) block. So far, the stabilization of the helical conformation of a poly- or copolypeptide was explained as a result of molecular weight effects.

In our case, we showed that the helical conformation of the poly(β-benzyl L-aspartate) (PBLA) blocks is stabilized by interactions with the poly(ethylene oxide) (PEO) segments, these interactions allowing the solubilization of the block copolymer in mixtures of $CHCl_3$/DMSO. The presence of such interactions was seen by ^1H and 2D ^1H,^1H NOESY NMR analysis using $CDCl_3$ and DMSO-d_6 as solvents. The helix−coil transition and the stability of the α-helix conformation were studied by specific rotation measurements in $CHCl_3$, DMSO, dichloroacetic acid (DCA), mixtures of $CHCl_3$/DMSO, and mixtures of $CHCl_3$/DCA.

Experimental Procedure

Synthesis. The synthesis and purification of the PEO/PBLA block copolymers were realized as reported previously.[25] Briefly, the PEO/PBLA block copolymers were obtained by ring-opening polymerization of the β-benzyl L-aspartate−*N*-carboxy anhydride (BLA−NCA) using the primary amino end group of the α-methoxy-ω-amino PEO. The polymerization was stopped when the peaks corresponding to the BLA−NCA (1850, 1790, and 915 cm^{-1}) disappeared from the IR spectrum. After purification by selective precipitation in 2-propanol in order to eliminate oligomers and homopolymers, the resulting block copolymers were studied by ^1H and 2D ^1H,^1H NOESY NMR as well as by specific rotation measurements in chloroform, dimethyl sulfoxide, and mixtures of the two solvents. Specific rotation was also measured in $CHCl_3$, DCA, and mixtures of $CHCl_3$/DCA.

Characterization. ^1H and 2D ^1H,^1H NOESY NMR. The PEO/PBLA block copolymer was dissolved in $CDCl_3$ or in DMSO-d_6 or in mixtures of $CDCl_3$/DMSO-d_6. ^1H and 2D ^1H,^1H NOESY NMR spectra were recorded on a JEOL EX400 (400 MHz) spectrometer using 5 mm NMR tubes. ^1H chemical shifts are reported in ppm with the solvent as the internal reference. The 2D ^1H,^1H NOESY NMR spectra were recorded at 400 MHz with sweep widths of 4000 Hz into 1024 data points. The pulse sequence used is the following: $90°-t_1-90°-\Delta-90°-$FID, where Δ is the mixing time. The first 90° pulse was 14 ms, the relaxation delay was 0.25 ms (t_1). The second 90° pulse was 14 ms, and the mixing time was 20 ms (Δ). After a third 90° pulse of 14 ms, FID was recorded with eight scans.

Optical Rotation Measurements. The specific optical rotation measurements were realized using a digital polarimeter DIP-370 (JASCO) at 546 nm wavelength, a cell of 100 mm length and an integration time of 30 s. The concentration in poly(amino acid benzyl ester) segments was 0.1 wt % for all the measurements. The block copolymer was dissolved in $CHCl_3$, DMSO, DCA, mixtures of x% $CHCl_3$/(100 − x)% DMSO ($0 \le x \le 100$), or mixtures of x% $CHCl_3$/(100 − x)% DCA ($0 \le x \le 100$). Before each measurement, a blank solution, having the same composition as the sample solution, was measured in order to obtain the "zero". Each sample was measured two times, and an average value is given.

Results and Discussion

The PEO/PBLA polymeric micelles are receiving an increasing amount of interest as potential drug carriers for drug delivery systems. Indeed, due to its particular structure made up by a hydrophobic inner core (PBLA) surrounded by a corona of hydrophilic chains (PEO), this kind of carrier can solubilize hydrophobic drugs in the inner core.[1−7] Because the polymeric micelles are prepared from an organic solution of the PEO/PBLA block copolymer and since some properties of the carrier might depend on the conformation of the poly(amino acid benzyl ester), it is important to characterize the conformation of the PBLA blocks and to determine the influence of the PEO segments on the conformation of the PBLA blocks.

Several methods were used to study the conformational structure of polypeptides and copolypeptides: circular dichroism (CD), optical rotatory dispersion (ORD), ^1H NMR, etc. Sederel et al.[26] studied the conformation of copolymers of β-benzyl L-aspartate with different poly(amino acids) in the solid state by IR spectroscopy and CD. These techniques allowed them to demonstrate that the incorporation of amino acid residues in the PBLA chains induces a change in the conformation of the PBLA from the left-handed into the right-handed α-helix. Kugo et al. also studied the conformation of the poly(γ-benzyl L-glutamate-*co*-ethylene oxide-*co*-γ-benzyl L-glutamate) triblock copolymers in the solid state. They showed by CD in the solid state that the helical content of the polypeptide block chain decreases gradually as it is swollen in water.[27]

However, even if these methods are efficient in the analysis of the solid state conformation of the polypeptide blocks, we were more interested in the solution properties of the PEO/PBLA block copolymers since micelle formation takes place in solution. One powerful method to study the conformation of the polypeptides and copolypeptides in solution is the high-resolution proton magnetic resonance (NMR). Bradbury et al. studied the conformation of water-soluble polypeptides[28] and of random copoly(benzyl L-glutamate-*co*-benzyl L-aspartate) and copoly(benzyl D-glutamate-*co*-benzyl L-aspartate)[29] by high-resolution NMR. They showed that the helix−coil transition can be followed by this analytical method.[28,29] They also reported that the sense of the α-helix of the PBLA residues can be determined from the α-C*H* and N*H* chemical shifts.[29] All the data obtained from NMR were in good agreement with those obtained from ORD.[28,29]

On the basis of this information, we studied our copolymers by ^1H NMR in deuterated chloroform and in DMSO-d_6. Although Ribeiro et al. reported that the NMR spctra of PEO-bound polypeptides in $CDCl_3$ are quantitatively similar to those of the corresponding low molecular weight peptides and that PEO segments have little effect on the peptides structure,[16,30] we found that the PEO segments induce changes in the structure of the PBLA blocks. Indeed, the comparison of the ^1H NMR spectrum in $CDCl_3$ of the PEO/PBLA block copolymer (Figure 1a) and of the mixture of the PEO and PBLA homopolymers (Figure 1b) gave evidence of these changes: the lateral methylene protons adjacent to the benzyl group of the PBLA segments appeared as a single peak at 5.05 ppm in the case of the mixture of homopolymers whereas it gave two doublets centered at 5.05 ppm and at 5.15 ppm for the PEO/PBLA block copolymer. On the other hand, in DMSO-d_6, these methylene protons gave only one narrow peak at 5.05 ppm (Figure 2). These results suggest that in chloroform, the PBLA segments adopt a particular structure induced by the presence of the PEO blocks. Since chloroform is known to be a helix-supporting solvent,[16,28,31] it was logical to think that in chloroform, the PBLA segments have an α-helix conformation.

In order to determine the stability of this structure in the mixture $CHCl_3$/DMSO, we realized a titration by ^1H NMR using the methylene protons adjacent to the benzyl group of the PBLA blocks (Figure 3). This titration showed that more than 40% of DMSO was

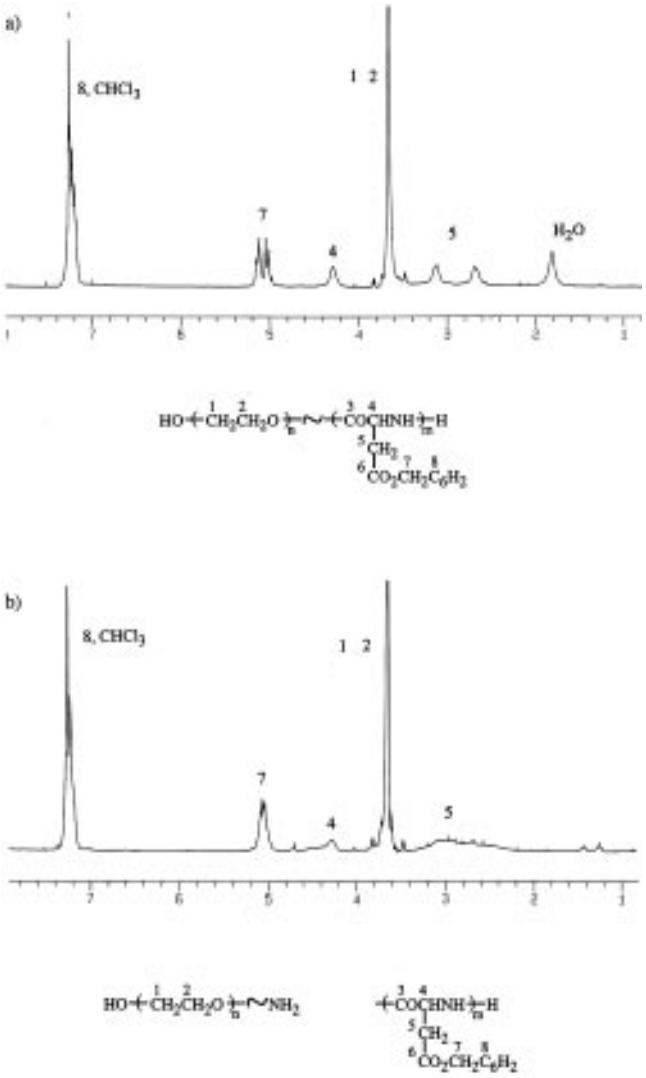

Figure 1. ^{1}H NMR (400 MHz) spectrum at 25 °C in $CDCl_3$ of (a) the PEO/PBLA block copolymer and (b) the mixture of PEO homopolymer + PBLA homopolymer (1/1).

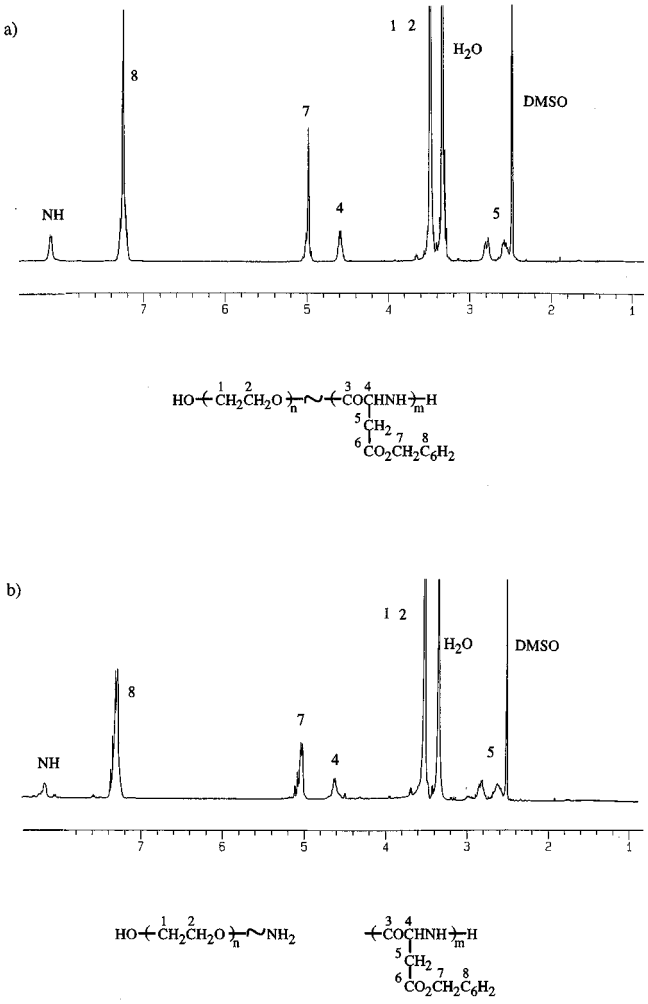

Figure 2. ^{1}H NMR (400 MHz) spectrum at 25 °C in DMSO-d_6 of (a) the PEO/PBLA block copolymer and (b) the mixture of PEO homopolymer + PBLA homopolymer (1/1).

necessary to observe a change in the structure of the PBLA blocks. In addition, the change in the conformation can be followed by the chemical shift of the backbone α-CH of the PBLA segments. Indeed, as shown by Figure 4, a shift in the chemical shift of this proton is observed with increasing DMSO content. According to Bradbury et al.,[29] a chemical shift of 4.30 ppm for the backbone α-CH corresponds to a PBLA having a helical conformation ($CDCl_3$), whereas a chemical shift of 4.70 ppm represents the random-coil conformation (DMSO).

In order to determine the sense of the α-helix conformation of the PEO/PBLA block copolymer in chloroform and to have a more precise idea of the stability of the helical conformation of the PBLA segments, we studied the block copolymer's solutions by optical rotation measurements at 546 nm wavelength. Indeed, Karlson et al.[32] reported the determination of the sense of the α-helix of PBLA homopolymer (molecular weight: 50 000) in chloroform by optical rotation measurements at 546 nm. They also measured the stability of the α-helix conformation of PBLA in chloroform and in mixtures of chloroform/dichloroacetic acid (DCA) by this method. They showed that PBLA adopts a left-handed α-helix in chloroform ($[\alpha]_{546} = -167.6°$) and that 8% of DCA was necessary to destabilize the left-handed α-helix and to observe a random-coil conformation ($[\alpha]_{546} = -19°$).[32,33]

As shown by Figure 5a,b, the PBLA blocks in our PEO/PBLA block copolymer adopt a left-handed α-helix conformation in $CHCl_3$ ($[\alpha]_{546} = -146.68°$) whereas, in DMSO and in DCA, the conformation of the PBLA blocks is a random coil ($[\alpha]_{546} = -37.44°$ in DMSO and $[\alpha]_{546} = -19°$ in DCA). These data are in good agreement with the results obtained from ^{1}H NMR spectra: the PBLA segments have an α-helix conformation in chloroform, whereas a random coil is observed in DMSO. The gradual increase of the optical rotation with increasing DMSO content gives evidence of the stability of the left-handed α-helix conformation of the PBLA segments in the PEO/PBLA block copolymers. The helix−coil transition occurs between 20% and 60% of DMSO (Figure 5a); a similar interval (20% to 60% of DMSO) for the helix−coil transition was obtained by the ^{1}H NMR titration (Figure 4). The left-handed α-helix conformation of PBLA segments in the PEO/PBLA block copolymers (molecular weight = 10 000) is stable in the chloroform/DMSO mixture. In this case, the stabilization of the α-helix conformation cannot be a consequence of the increase in molecular weight since the molecular weight of the block copolymer is lower than the minimal reported molecular weight for PBLA to observe the α-helix conformation (about 50 000). Further, we assumed that the stabilization of the α-helix conformation was a result of interactions between the two blocks. Conversely, we showed that less than 5% of DCA was necessary to destabilize the α-helix conformation of the PBLA blocks and to observe a random-coil conformation

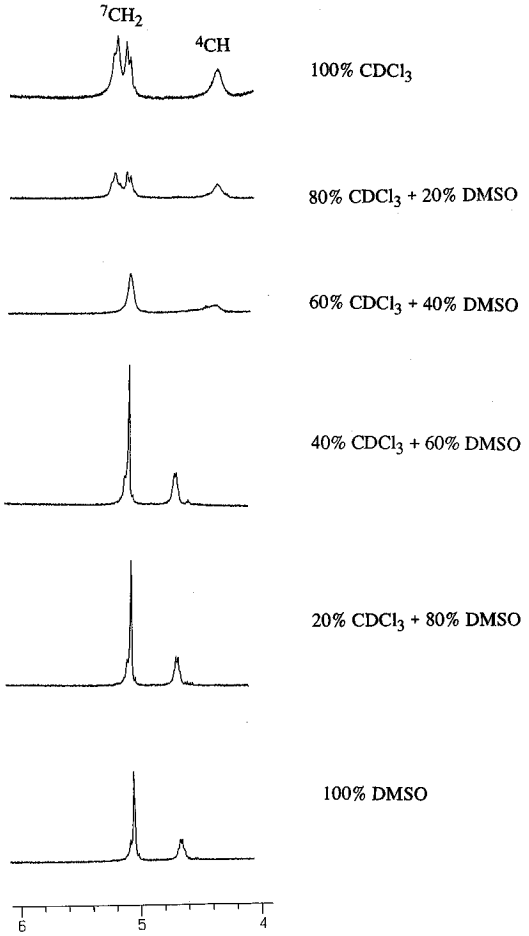

Figure 3. ^1H NMR (400 MHz) spectra in $CDCl_3$, in DMSO-d_6, and in mixtures of x% $CDCl_3$/(100 − x)% DMSO-d_6 ($0 \leq x \leq 100$) of the methylene protons adjacent to the benzyl group ($^7CH_2$) of the PBLA segments in the PEO/PBLA block copolymer at 25 °C.

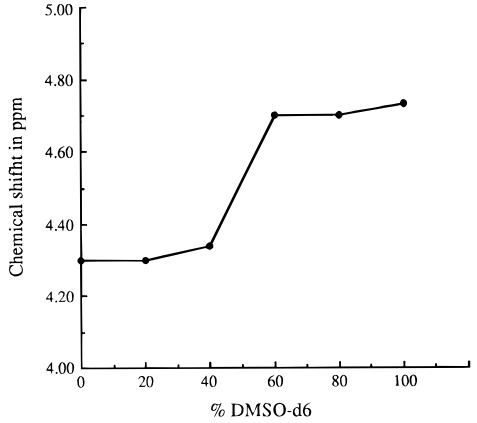

Figure 4. Chemical shifts of the backbone α-CH (^4CH) of the PBLA segments as a function of the % of DMSO-d_6.

(Figure 5b). Since DCA is able to destroy hydrogen bonds between chains, we speculated that the interactions that stabilized the left-handed α-helix in $CHCl_3$ and allowed the solubilization of the block copolymers in mixtures of $CHCl_3$/DMSO are of the hydrogen bond type. As will be described below, we demonstrated this hypothesis by using the high-resolution NMR technique.

2D ^1H,^1H NOESY NMR spectroscopy appears to be a powerful method to investigate the geometrical structure of molecules. Usually, the two-dimensional NMR methods are based on the coupling between nuclear dipoles, these couplings being between nuclear dipoles in the same molecule. However, as in the Overhauser

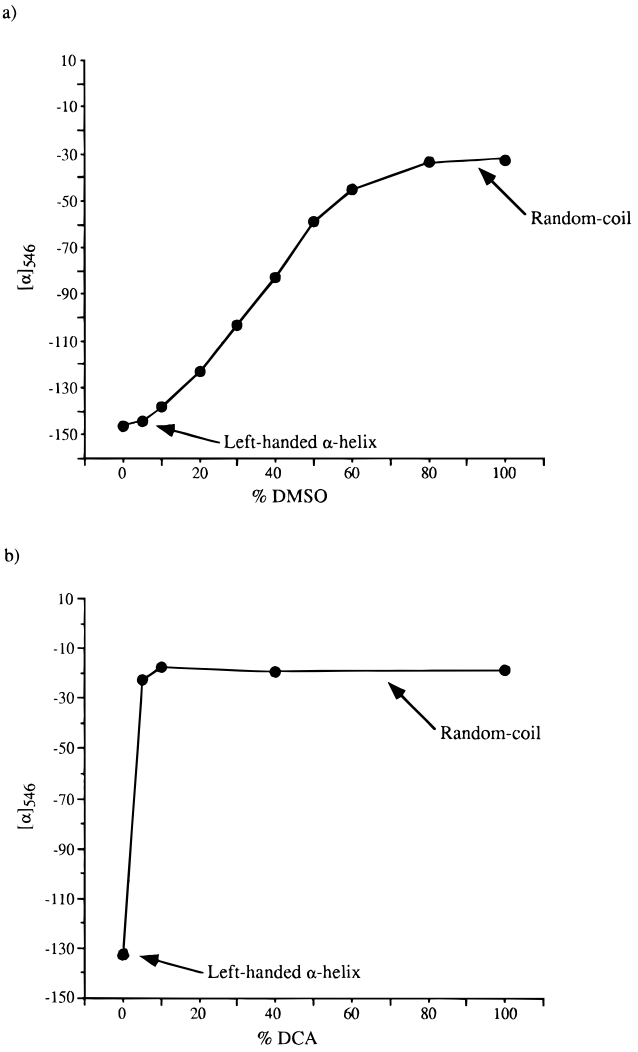

Figure 5. Optical rotation measurement at 546 nm wavelength in (a) $CHCl_3$, DMSO, and mixtures of x% $CHCl_3$/(100 − x)% DMSO ($0 \leq x \leq 100$) and (b) $CHCl_3$, DCA, and mixtures of x% $CHCl_3$/(100 − x)% DCA ($0 \leq x \leq 100$).

effect, the interactions may also be of a dipolar type, i.e., through the space. The 2D ^1H,^1H NOESY NMR has been successfully used for determining the structure of peptides, proteins, and oligosaccharides.[34] As shown by Figure 6a, the 2D ^1H,^1H NOESY NMR spectrum in $CDCl_3$ of the block copolymer gave evidence of interactions between the methylene protons ($CO_2CH_2C_6H_5$) of the PBLA chains and the methylene protons of the PEO segments as well as between the benzyl protons of the PBLA blocks and the methylene protons of the PEO chains. Such interactions are not observed in the 2D ^1H,^1H NOESY NMR spectrum of the block copolymer in DMSO-d_6; only interactions between the protons of the two segments and the water contained in the block copolymer and/or in the solvent (peak at 3.3 ppm) are shown by this two-dimensional spectrum (Figure 6b). Thus, these results and the results obtained from specific rotation measurements proved that the left-handed α-helix conformation of PBLA blocks in the PEO/PBLA block copolymers is stabilized by interactions of the hydrogen bond type between the two blocks, these interactions allowing the solubilization of the block copolymers in mixtures of $CHCl_3$/DMSO in which the PBLA homopolymer is not soluble.

At last, the ORD spectrum of the block copolymer in chloroform showed that the PBLA segments have a left-handed α-helix in this solvent (data not shown). The

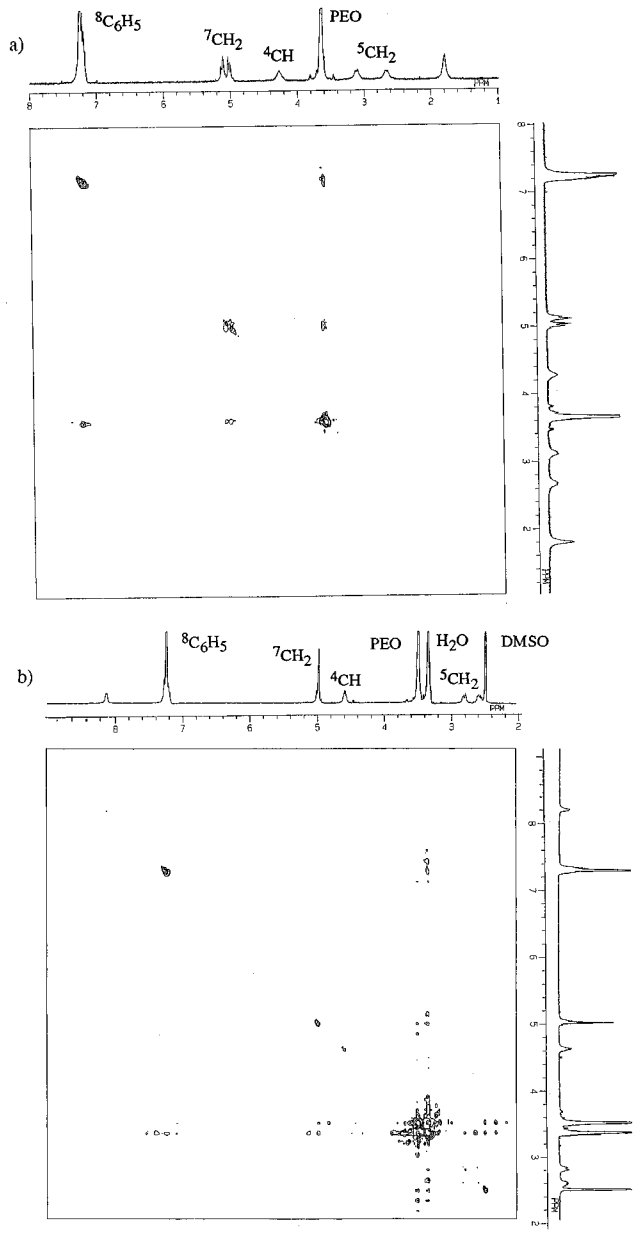

Figure 6. 2D $^1H,^1H$ NOESY NMR spectrum of the PEO/PBLA block copolymer in (a) $CDCl_3$ and (b) DMSO-d_6.

result is in good agreement with all the previous conclusions.

Conclusion

From the data presented in this study, we can conclude that the α-helix conformation of the PBLA segments in the PEO/PBLA block copolymers can be stabilized not only by increasing the molecular weight but also by intramolecular interactions between the two blocks. The PEO chains have the capacity to induce and stabilize the helical conformation of PBLA in organic solvents. We assumed that these interactions are of the hydrogen bonding type. Recently, we also described the stabilization of the α-helix conformation of poly(L-lysine)-bound PEO in aqueous medium as a result of intramolecular interactions between the two blocks. In this case, the interactions were assumed to be of a hydrophobic nature.[35]

The results gathered in this paper are investigated not only for a theoretical point of view but also for the comprehension of the PEO/PBLA micelles formation. Indeed, since these micelles are formed from an organic solution, the knowledge of the PBLA segments conformation is important in order to control the properties of the resulting micelles. At the present time, the influence of the conformation of the PBLA segments on the formation and on the properties of the micelles is under investigation and will be published elsewhere.

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