Diffusional Behavior of Polypeptides in the Isotropic, Biphasic, and Liquid Crystalline Phases As Studied by the Pulse Field-Gradient Spin−Echo ^{1}H NMR Method

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ABSTRACT: The self-diffusion coefficients (*D*) of α-helical poly(*n*-octadecyl L-glutamate) (POLG) and chloroform ($CHCl_3$) as solvent in the isotropic, biphasic, and liquid crystalline phases were measured as a function of POLG concentration at 30 °C by the pulse field-gradient spin-echo ^{1}H NMR method and the diffusion ^{1}H NMR imaging method, to elucidate diffusional behavior of the polypeptide and solvent. From the experimental results, it was found that the diffusion coefficient of POLG is largely decreased in going from the isotropic phase to the liquid crystalline phase, and in the biphasic phase two kinds of fast and small diffusion components with diffusion coefficients of $\sim 10^{-6}$ and $\sim 10^{-7}$ cm^2/s, respectively, exist, which correspond to the isotropic and liquid crystalline regions, respectively. Further, it was found that in the liquid crystalline phase the diffusion coefficients of chloroform as solvent in the liquid crystalline phase in the directions parallel ($D_{\parallel}$) and perpendicular ($D_{\perp}$) to the α-helical chain axis are different from each other and then $D_{\parallel}$ is much larger than $D_{\perp}$.

Introduction

In our previous works,[1,2] we have successfully measured the diffusion coefficients of rodlike polypeptides such as α-helical poly(γ-glutamates) having long *n*-alkyl side chains as a function of the α-helical chain length in the thermotropic liquid crystalline state by high field-gradient ^{1}H NMR method, which form by melting of *n*-alkyl side chain crystallites.[3−8] From these experimental results, it has been shown that the diffusion of the rodlike polypeptide chain is anisotropic and follows the Kirkwood theory of rodlike polymers[9,10] and that high field-gradient NMR is a very powerful means for determining very small diffusion coefficient for polypeptide systems.

It is well-known that poly(glutamate) systems such as poly(γ-benzyl L-glutamate), poly(γ-*n*-alkyl L-glutamate), etc., in solvent form the isotropic, biphasic, and liquid crystalline phases, depending on the polypeptide concentration.[11−19] However, in these phases the diffusional behavior of the polypeptides is not clarified.

From such a background, in this work, we aim to elucidate the diffusional behavior of α-helical poly(γ-*n*-octadecyl L-glutamate) and chloroform as solvent in the isotropic, biphasic, and liquid crystalline phases by using high field-gradient spin-echo (PFGSE) ^{1}H NMR and diffusion ^{1}H NMR imaging.

Experiment

A. Materials. Poly(γ-*n*-octadecyl L-glutamate) (POLG) was synthesized by ester-exchange reactions between poly(γ-benzyl L-glutamate) (PBLG) (average molecular weight: $M_w = 30\,000$) and *n*-octadecyl alcohol. The reaction was carried out in 1,2-dichloroethane with *p*-toluenesulfonic acid as a catalyst at 60 °C. The complete replacement of the phenyl groups of PBLG by *n*-alkyl groups was confirmed by their ^{1}H NMR spectra recorded in solution.

The POLG solution was prepared by placing POLG and deuterated chloroform ($CDCl_3$) in an NMR tube with a diameter of 5 mm, which is used as solvent for diffusion coefficient measurements of POLG, and its NMR tube was sealed off and was kept at 50 °C. Protonated chloroform ($CHCl_3$) is used for diffusion coefficient measurement of chloroform in the POLG solution. The POLG concentrations employed in this work were 5, 10, 15, 17.5, and 20% w/w. The 5 and 10% w/w POLG solutions are in the isotropic phase, the 15 and 17.5% w/w POLG solutions are in the biphasic phase, and the 20% w/w POLG solution is in the cholesteric liquid crystalline phase as seen from phase diagram.[19] When the 20% w/w POLG solution is placed in a 13.5 T NMR magnet for 80 h, it forms a highly oriented nematic liquid crystalline phase. The degree of order parameter is 0.86 as determined by static ^{13}C NMR.[20,21]

B. Measurements. The self-diffusion coefficient measurements of POLG in the lyotropic liquid crystalline state were carried out by means of a JEOL GSX-270 NMR spectrometer operating at 270.1 MHz for ^{1}H at 30 °C with a homemade pulse gradient generator using a standard PFGSE pulse sequence (the Hahn echo sequence: $\pi/2$ pulse−τ−π pulse)[22−24] with field gradient pulse between the $\pi/2$ and π pulses and between the π and spin-echo.[25−37] In this work, a field-gradient strength of about 13.1 T m^{-1} (1310 G/cm) was used.[37−42] The spectral width was 4.0 kHz and data points were 4096. The relationship between the echo signal intensity and pulse field gradient parameters is given by

$$A(\delta)/A(0) = \exp[-\gamma^2 G^2 D \delta^2 (\Delta - \delta/3)] \qquad (1)$$

where $A(\delta)$ and $A(0)$ are echo signal intensities at $t = 2\tau$ with and without the magnetic field gradient pulse, respectively. The field gradient pulse width is δ. τ is the pulse interval, γ the gyromagnetic ratio of proton, G the field-gradient strength, D the self-diffusional coefficient, and Δ the field gradient pulse interval. The echo signal intensity was measured as a function of δ. The plot of $ln\ [A(\delta)/A(0)]$ against $\gamma^2 G^2 \delta^2 (\Delta - \delta/3)$ gives a straight line with a slope of $-D$. The τ, Δ, and δ values employed in these experiments were 4, 4, and 0.001−0.4 ms, respectively. The diffusion coefficient D of water of 2.5×10^{-5} cm^2/s at 303 K was used as the calibration of the field-gradient strength as well-known.[36] The experimental error for the D value was estimated to be within 5%.

As probe molecules in the biphasic phase have two-diffusion components in diffusion on the measurement time scale, the

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total echo attenuation is given by a superposition of contributions from the individual components as expressed by

$$A(\delta)/A(0) = f_1 \exp[-\gamma^2 G^2 D_1 \delta^2 (\Delta - \delta/3)] + f_2 \exp[-\gamma^2 G^2 D_2 \delta^2 (\Delta - \delta/3)] \quad (2)$$

where D_i is the self-diffusion coefficient of the ith component, and f_i is the fraction of the ith component and thus $f_1 + f_2 = 1$. The fraction for the fast and slow diffusion components can be determined from the intercept of the least-squares fitted straight line.

^{1}H NMR imaging measurements on diffusion coefficient of chloroform solvent in POLG/CHCl$_3$ liquid crystalline solution were carried out by means of a Bruker Avance DSX300 NMR spectrometer operating at 300.13 MHz with an accessory of diffusion imaging system with field gradient of 100 G/cm at 30 °C. The diffusion imaging pulse sequence is based on the spin-echo sequence including field gradient pulses.[25−27] The slice selection is achieved with the field gradient in the z or x direction. In an NMR magnet, POLG chains in the liquid crystalline solution are oriented to the magnetic field that is the z direction, after an NMR tube containing the liquid crystalline solution is placed within for a long time. Thus, by the slice selection in the z direction, the parallel diffusion coefficient of CHCl$_3$ ($D_{\|}$) in direction parallel to the α-helical POLG chain axis in POLG lyotropic liquid crystalline solution is determined, and the perpendicular diffusion coefficient ($D_{\perp}$) by the slice selection in the x direction perpendicular to the α-helical POLG chain axis is determined. The values of Δ and δ are 10 ms and 0.001−0.1 ms, respectively.

The ^{1}H chemical shifts for the POLG and CHCl$_3$ are calibrated relative to tetramethylsilane (TMS).

Results and Discussion

A. Diffusional Behavior of POLG in the Isotropic, Biphasic, and Liquid Crystalline Phases. Figure 1 shows typical PFGSE ^{1}H NMR spectra of POLG solutions in the isotropic, biphasic, and liquid crystalline phases[44] at various POLG concentrations at 30 °C as a function of field-gradient pulse length (δ). In the PFGSE ^{1}H NMR spectra of 5% w/w POLG solution in the isotropic phase as shown in Figure 1a, an asymmetric sharp signal and a shoulder peak appear at about 1.4 and 1 ppm, respectively. The intense peak and the shoulder peak can be straightforwardly assigned to the internal methylene protons and the terminal methyl protons in the side chains, respectively. The peaks for the main-chain protons such as the α-CH and amide protons do not appear by great line-broadening because of the slow molecular motion in the ^{1}H NMR time scale within the measurement temperature. The spectrum does not appear peak from protonated chloroform as impurity in deuterated chloroform as solvent. Figure 1b shows typical PFGSE ^{1}H spectra for a 20% w/w POLG solution in the liquid crystalline phase at 30 °C. It is noted that the signal becomes much broader as compared with that in the isotropic phase, and the signal intensity for the former is much more slowly reduced with an increase in field-gradient pulse duration (δ) as compared with the latter as shown in Figure 1a. This is due to a large reduction of diffusion rate in the liquid crystalline phase.

By using these decay signals, the diffusion coefficient D of POLG in solution can be determined from the slope of the plots of ln[$A(\delta)/A(0)$] against $\gamma^2 G^2 \delta^2(\Delta - \delta/3)$ as shown in Table 1. As shown in Figure 2, parts a and b, the plots of ln[$A(\delta)/A(0)$] for 5 and 10% w/w POLG solutions against $\gamma^2 G^2 \delta^2(\Delta - \delta/3)$ remain a straight line. This shows that the diffusion of POLG becomes a single diffusion component. Then, the diffusion coefficients D for α-helical POLG in 5 and 10% w/w POLG solutions which are in the isotropic phase are obtained as shown in this table.

In 15 and 17.5% w/w POLG solutions which are in the biphasic phase, the plots of ln[$A(\delta)/A(0)$] against $\gamma^2 G^2 \delta^2(\Delta - \delta/3)$ consist of two straight lines with different slope as shown in Figure 2, parts c and d. This shows that the diffusion is made up of two kinds of diffusion components such as the slow diffusion component and fast diffusion component. The diffusion coefficients D for the slow diffusion component and fast diffusion component were determined from the slopes as shown in Table 1. The fast diffusion component and slow diffusion component in the biphasic phase correspond to the isotropic region and the liquid crystalline region, respectively. The D value for the fast diffusion component is very close to the D values for 5 and 10% w/w POLG solutions in the isotropic phase as shown in Table 1. On the other hand, the diffusion coefficient of the slow diffusion component is very close to that for 20% w/w POLG solution in the liquid crystalline phase. These results mean that the diffusion processes for the isotropic region and the liquid crystalline region in the biphasic phase are very similar to those in the isotropic phase and in the liquid crystalline phase, respectively. The fractions of the two kinds of diffusion components can be determined by analyzing the plots of ln[$A(\delta)/A(0)$] against $\gamma^2 G^2 \delta^2(\Delta - \delta/3)$ with two straight lines as shown in Figure 2, parts c and d. As for the 15% w/w POLG solution, the fractions of the diffusion components for the isotropic and liquid crystalline regions in the biphasic phase are determined to be 62 and 38%, respectively, and for 17.5% w/w POLG solution to be 31 and 69%, respectively. The fraction of the fast diffusion component corresponding to the isotropic region is largely decreased despite small concentration increase in going from 15% w/w POLG concentration to 17.5 w/w POLG concentration. Parts a, b and e of Figure 2 show the first data point somewhat higher than the straight line indicates. We think that it does not come from a faster-moving species. It may be an artifact. At this stage, it is not conclusive.

In 20% w/w POLG solution which is in the liquid crystalline phase, as shown in Figure 2e, the plots of ln[$A(\delta)/A(0)$] for α-helical POLG against $\gamma^2 G^2 \delta^2(\Delta - \delta/3)$ remain a straight line. This shows that the diffusion of POLG becomes a single diffusion component. Then, the diffusion coefficient for α-helical POLG in 20% w/w POLG solution is obtained. This value is very close to that for the liquid crystalline region in the biphasic phase. The determined D value is listed in Table 1. For example, the diffusion coefficient as determined from Figure 2a is 2.76×10^{-6} cm^2/s. This diffusion for a polypeptide chain with 200 Å is very fast. Probably such a fast diffusion comes from the fluidity of the liquid crystal.

In Figure 3, the determined D values are plotted against the POLG concentration in order to understand diffusional behavior of α-helical POLG in the isotropic, biphasic, and liquid crystalline phases. From this figure, it is seen that the diffusion coefficient of α-helical POLG chain in the isotropic region in the isotropic and biphasic phases decreases with an increase in the POLG concentration and transitionally decreasesd in going from the isotropic region to the liquid crystalline region. The diffusion coefficient of α-helical POLG chain in the

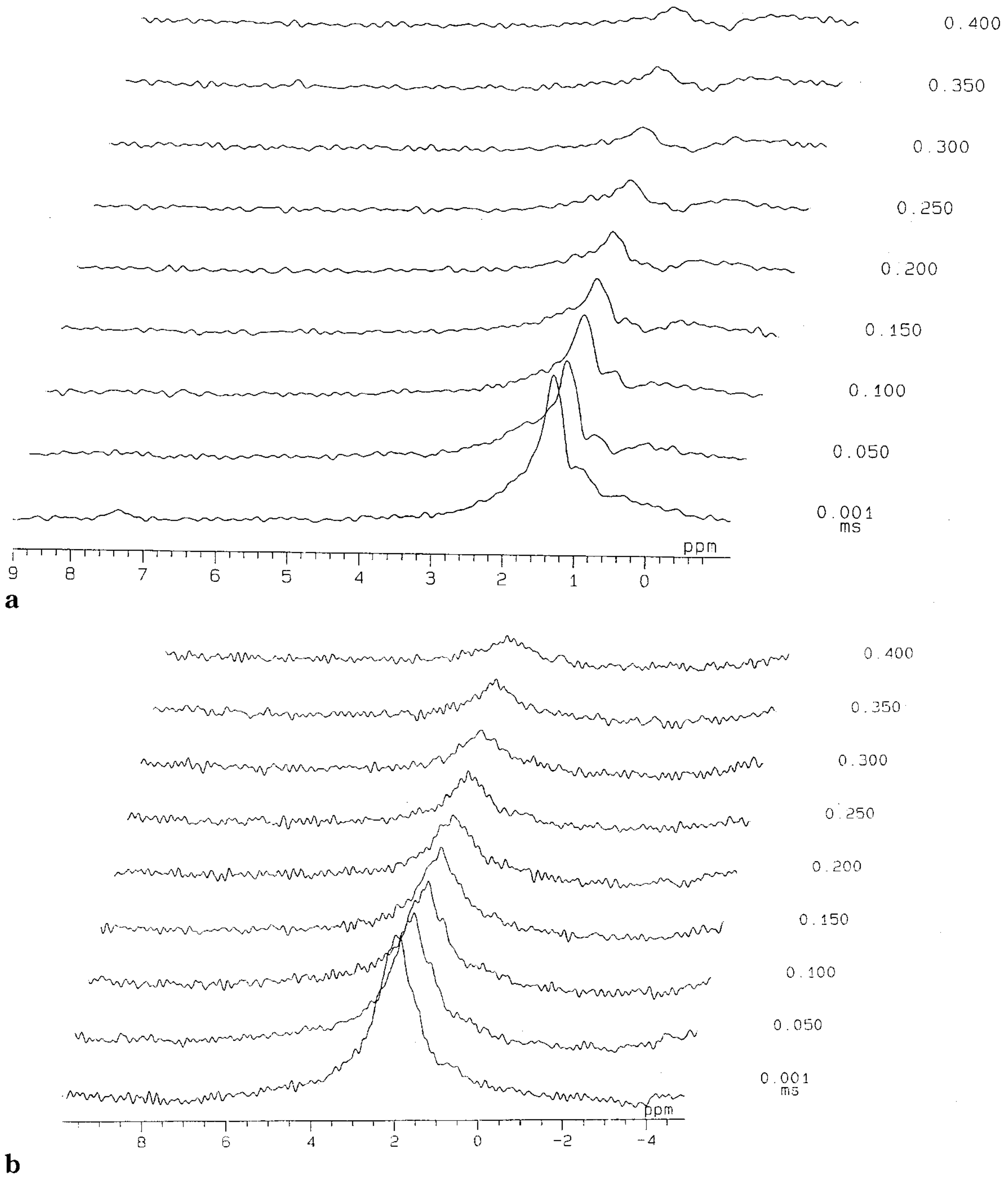

Figure 1. Typical PFGSE ^{1}H NMR spectra of POLG in 5 (a) and 20% w/w (b) POLG/deuterated chloroform solutions as a function of field-gradient pulse length δ at 30 °C.

Table 1. Determined Diffusion Coefficients *D* of POLG in POLG/Deuterated Chloroform Solutions at Various POLG Concentrations

	diffusion coeff D (10^{-7} cm^2/s)				
region	[POLG] = 5 wt %	[POLG] = 10 wt %	[POLG] = 15 wt %	[POLG] = 17.5 wt %	[POLG] = 20 wt %
liquid crystalline			6.83	6.34	5.94
isotropic	27.6	26.3	23.7	21.5	

liquid crystalline region is slightly decreased with an increase in the POLG concentration.

As reported previously,[1] POLG and POLLG (poly(γ-oleyl L-glutamate)) with the same α-helical chain length as POLG sample used in this work forms thermotropic liquid crystalline phase above 60 °C and above 30 °C, respectively. The $D_{\rm iso}$ values for POLG at 60 °C and that for POLLG in the thermotropic liquid crystalline phase at 30 °C were determined to be 3.53×10^{-7} and 4.94×10^{-7}cm^2/s, respectively. The $D_{\rm iso}$ for POLG in the liquid crystalline phase at 30 °C is much larger than these $D_{\rm iso}$ values for POLG at 60 °C and for POLLG at 30 °C. This may be explained by the Kirkwood theory as shown below.

The derivation of obtaining the translational diffusion coefficient of rodlike polymers derived by Doi and Edwards[9] on the basis of the Kirkwood theory,[10] the isotropic diffusion coefficient $D_{\rm iso}$ of a rodlike polymer

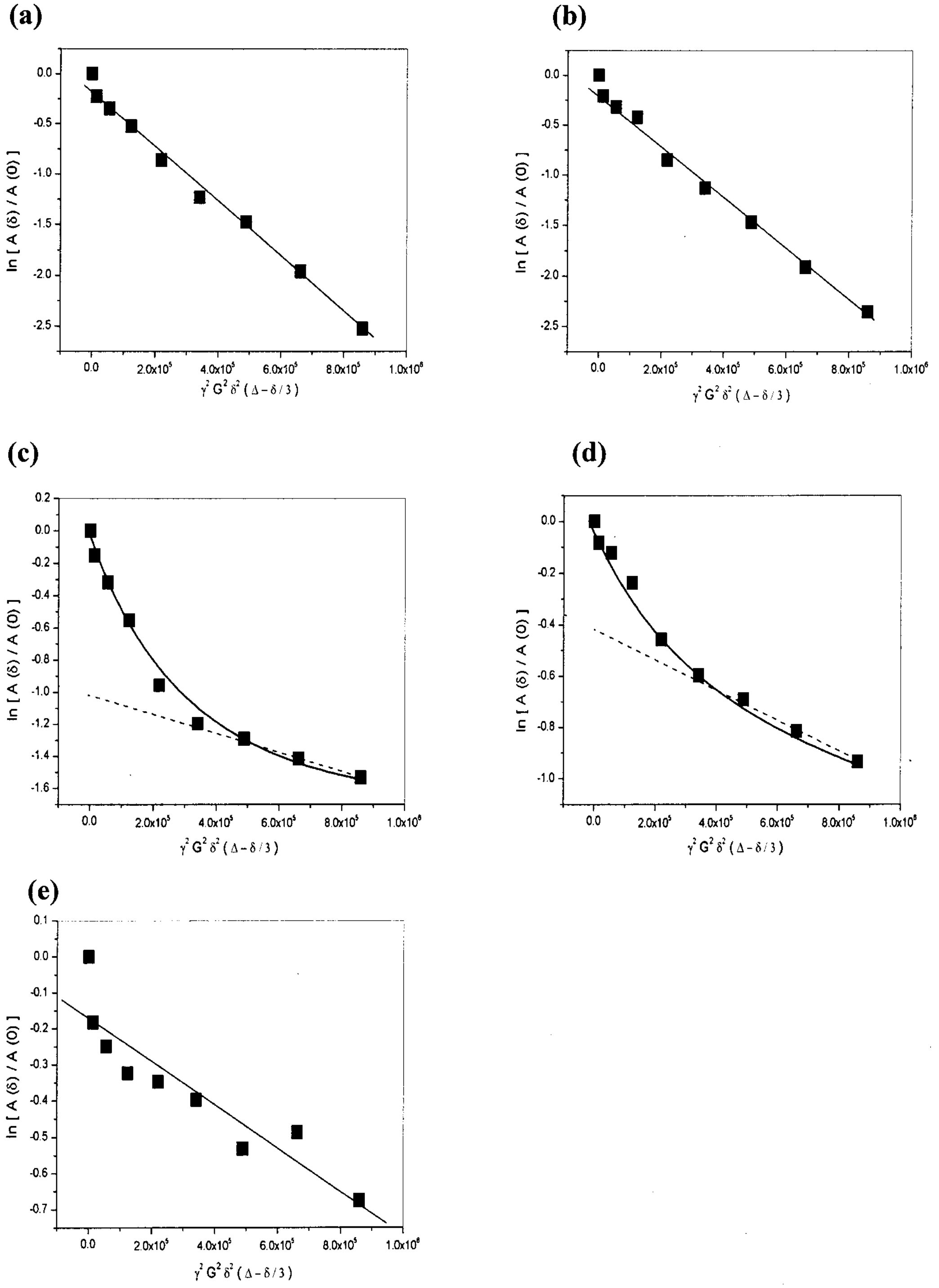

Figure 2. Plots of $\ln[A(\delta)/A(0)]$ against $\gamma^2G^2\delta^2(\Delta - \delta/3)$ for determining diffusion coefficient D of POLG in 5 (a), 10 (b), 15 (c), 17.5 (d), and 20% w/w (e) POLG/deuterated chloroform solutions at 30 °C.

chain is followed by $D_{\rm iso} = [\ln(L/b)/L]kT/3\pi\eta_s$ in which L is the rodlike polymer length, b is the diameter of the rodlike polymer, η_s is the viscosity of the solvent corresponding to long n-alkyl side chains in the thermotropic liquid crystalline state, k is the Boltzmann constant, and T is the absolute temperature. Using the standard bond lengths and bond angles determined by X-ray diffraction, we can straightforwardly estimate the rodlike main chain length and the diameter of α-helical polypeptides. Then, the main-chain lengths of α-helical polypeptide with average molecular weigths of 30 000 can be estimated to be L = ca. 200 Å, respectively, and the diameter of the α-helical main-chain including the ester group of side chain to be b = ca. 10 Å. The diffusion process of these rodlike polypeptides is expected to follow $D_{\rm iso} = [\ln(L/b)/L]\ kT/3\pi\eta_s$. This equation shows that the plots of $D_{\rm iso}$ against $\ln(L/b)/L$ become a straight line.

In the thermotropic liquid crystalline state, by melting of long n-alkyl side chain crystallites, the side chains play a role solvent in liquid crystal system. It is suggested that the difference in $D_{\rm iso} = [\ln(L/b)/L]kT/3\pi\eta_s$ between the thermotropic liquid crystalline phase and the lyotropic liquid crystalline phase is only η_s. It is apparent that η_s for the lyotropic liquid crystalline phase is much smaller than that for the thermotropic liquid

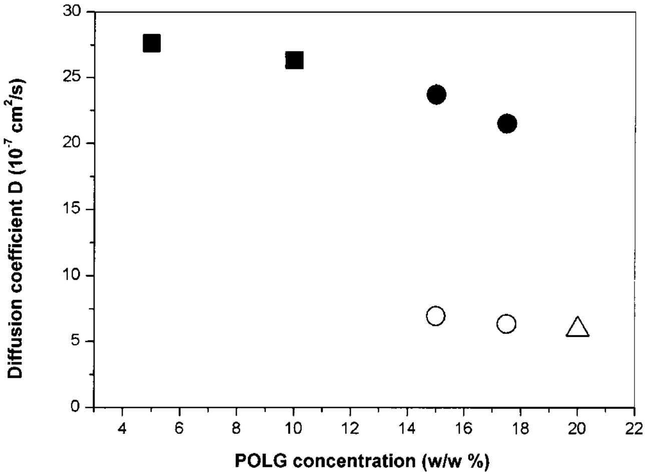

Figure 3. Plots of diffusion coefficients D of POLG in POLG/deuterated chloroform solutions against the POLG concentration at 30 °C: (■) 5 and 10% w/w POLG concentration (the isotropic phase); (●) 15 and 17.5% w/w POLG concentration (isotropic region in the biphasic phase); (○) 15 and 17.5% w/w POLG concentration (liquid crystalline region in the biphasic phase; (△) 20% w/w POLG concentration (the liquid crystalline phase).

crystalline phase at 30 °C. The difference in η_s may lead to the experimental fact that the D_{iso} for POLG in the lyotropic liquid crystalline phase is much larger than the D_{iso} values for POLG and for POLLG in the thermotropic liquid crystalline phase.

B. Diffusional Behavior of Chloroform Solvent in Highly Oriented POLG Liquid Crystalline Solution. To clarify the anisotropic diffusional behavior of $CHCl_3$ solvent in the lyotropic liquid crystalline system, polypeptide nematic liquid crystalline solutions with α-helical POLG chain highly oriented to the magnetic field in an NMR magnet were prepared. One of the best choices for anisotropic diffusion coefficient measurements in the highly oriented lyotropic liquid crystalline system is the use of NMR imaging because it can generate the field-gradient pulses in the x, y, and z directions. Thus, the diffusion coefficient of $CHCl_3$ solvent in the directions parallel ($D_{\parallel}$) and perpendicular ($D_{\perp}$) to the α-helical POLG chain axis was reliably determined by using ^{1}H NMR imaging equipment with a field gradient strength of 100 G/cm. However, this field gradient strength is not enough to determine the anisotropic diffusion coefficient of POLG in the liquid crystalline solution. For this reason, we are concerned with the anisotropic diffusion of $CHCl_3$ solvent in the liquid crystalline solution. The three-dimensional profiles for diffusion image in the x and z directions were measured as a function of field-gradient pulse length δ (not shown). By using the image profiles, the diffusion coefficients $D_{\parallel}$ and $D_{\perp}$ of chloroform solvent in the POLG nematic liquid crystal solution were determined from the plots of $\ln[A(\delta)/A(0)]$ against $\gamma^2G^2\delta^2(\Delta - \delta/3)$. Moreover, the diffusion coefficients of chloroform in the neat liquid state and in 20% w/w unoriented POLG/chloroform cholesteric liquid crystalline solution were determined by using the same method. The diffusion coefficient measurement on the cholesteric liquid crystalline solution was carried out immediately after the NMR sample tube was placed in an NMR magnet. At that time, the POLG chain is still unoriented to the magnetic field. The diffusion coefficient of $CHCl_3$ in the neat liquid state, the diffusion coefficient D_{iso} of $CHCl_3$ in the POLG liquid crystalline phase unoriented in the magnetic field, and the averaged diffusion coefficient $(D_{\parallel} + 2D_{\perp})/3$ of $CHCl_3$ in the POLG liquid crystalline phase highly oriented in the magnetic field as determined from the above experiments are listed in Table 2.

Table 2. Determined Diffusion Coefficients D of Chloroform in 20% w/w POLG/Chloroform Solution

	diffusion coeff D (10^{-5} cm^2/s)				
solvent	neat liquid	D_{iso}[a]	$D_{\parallel}$	$D_{\perp}$	$(D_{\parallel} + 2D_{\perp})/3$[b]
chloroform	2.55	1.82	1.61	1.18	1.32

[a] The diffusion coefficient measurement on the cholesteric liquid crystalline solution was carried out immediately after the POLG sample tube was placed in an NMR magnet. At that time, the POLG chain is still unoriented to the magnetic field. [b] Averaged over anisotropic diffusion coefficients of $CHCl_3$ in POLG liquid crystalline phase highly oriented in the magnetic field.

As shown in Table 2, the determined diffusion coefficient of chloroform in neat liquid is larger than that in 20% POLG/chloroform cholesteric liquid crystalline solution at 30 °C. This means that the diffusion of chloroform in the liquid crystalline solution is restrained by intermolecular interaction with POLG chains. After an NMR tube containing 20% POLG/chloroform cholesteric liquid crystalline solution was placed in a magnet with 13.5 T for 80 h, the POLG chains are highly oriented to the magnetic field, and the solution forms a nematic liquid crystalline solution. By application of the field-gradient pulse from the directions parallel and perpendicular to the POLG chain axis to the highly oriented POLG/chloroform liquid crystalline solution the diffusion coefficients $D_{\parallel}$ and $D_{\perp}$ for chloroform were determined to be 1.61×10^{-5} and 1.18×10^{-5} cm^2/s, respectively, as shown in Table 2, and the rate is ca. 1.4. This shows that the diffusion of chloroform in the direction parallel to the α-helical POLG chain axis is much faster than that perpendicular to the α-helical POLG chain axis. The diffusion process of chloroform in highly oriented POLG field is anisotropic. This result is very similar to the previous work[20,21] where the diffusion of dioxane solvent and probe n-alkanes in highly oriented poly(γ-benzyl L-glutamate) (PBLG) gel in the direction parallel to the α-helical PBLG chain axis is much faster than that in the direction perpendicular to the α-helical PBLG chain axis and thus the diffusion process is anisotropic. Such a behavior has appeared in the diffusion of solvent in lyotropic low-molecular weight liquid crystal systems.[43]

Conclusions

We can conclude as follows. The diffusion coefficients of α-helical POLG chain in the isotropic, bipasic, and liquid crystalline phases were successfully measured. It is shown that the diffusion of α-helical POLG chain in the liquid crystalline region is much slower than that in the isotropic region and that the diffusion of chloroform as solvent in the direction parallel to the α-helical POLG chain axis is much faster than that perpendicular to the α-helical POLG chain axis and thus the diffusion process of chloroform in highly oriented POLG field is anisotropic.

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