ESR Spin Probe Investigation of Chain Ordering of a Triglycerol Membrane

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The chain ordering of a triglycerol membrane was investigated by continuous wave (CW) electron spin resonance (ESR) in conjunction with a spin probe method. Order parameters (S_0) obtained by a slow-tumbling computational analysis for 7-doxylstearic acids (7-DSA), 12-DSA, and 16-DSA in poly(oxyethylene) hydrogenated castor oil (HCO) membrane at 20 °C were 0.68, 0.25, and 0.04, respectively. The S_0 results suggest that the membrane chains are well ordered around the probe moiety for 7-DSA and very fluid around the 16-DSA moiety in the HCO membrane. The relative magnitude of the chain order for 12-DSA was very different from that obtained by the conventional order parameter using hyperfine coupling values. The conventional hyperfine analysis cannot verify the subtle spectral changes for 12-DSA. Moreover, the S_0 values for 7- and 12-DSA decreased differently when the temperature increased. Thus, the results obtained by a slow-tumbling simulation revealed detailed membrane chain ordering.

ESR (electron spin resonance) in conjunction with the spin probe method has considerable advantage in the study of membrane behaviors.[1] The macroscopic and local viscosity of the environment profoundly influences the rate of lipid molecular reorientation. The behavior of the lipid molecule in the membrane is strongly influenced by the temperature. Changes in the viscosity are reflected in the ESR line width as well as the line shape because of anisotropy in the g value and in the ^{14}N hyperfine structure. The conventional order parameter[2] calculated using the hyperfine coupling values gives limited information regarding the chain ordering of the membranes. Subtle changes in the ESR line shape do not always reflect the hyperfine coupling values. The line shape of the ESR signal can also be analyzed by a computational method to determine the probe dynamics, such as chain ordering.[3] A computational analysis of the ESR spectra gives qualitative information concerning the membrane behaviors. Thus, an ESR investigation of the thermal behavior of the membrane chain provides details regarding the membrane characteristics and dynamics.

Poly(oxyethylene) hydrogenated castor oil (termed HCO) has a unique chemical structure of triglycerol. It consists of a uniform triglycerol structure embodying oxyethylene groups. Most naturally occurring triglycerols are mixed triglycerols; that is, the three fatty-portions of the glycerol are not the same. Thus, HCO is very suitable for investigating the detailed motions of the lipid molecule as a function of the temperature. In addition to the chemical structure, HCO is physiologically nontoxic and a naturally degradable substance. However, the physicochemical characteristics of molecular aggregates for the nonionic amphiphilic compounds have not been well understood.

In a previous ESR investigation using various aliphatic spin probes,[4,5] the spin-lattice relaxation time (T_{1e}) became shorter when the position of the probe moved toward the inner membrane. The shorter T_{1e} implies more motional interaction between the probe and the membrane chain. In addition, the T_{1e} values of the probes supported perpendicular diffusion coefficients obtained by a spectral simulation.[5] The spin dynamics were also consistent with the observed ESR line shape.

In the present investigation, the chain ordering of aliphatic spin probes in aqueous dispersions of HCO was studied using ESR spectroscopy. A conventional hyperfine analysis and a slow-motional spectral simulation were used to extract information regarding the detailed chain ordering. The slow-motional simulation provides an order parameter (S_0) along with two rotational dynamic parameters, parallel and perpendicular rotational diffusion coefficients.[6–8] The qualitative S_0 values together with the two dynamic parameters at the different positions of the membrane are discussed in comparison with the conventional hyperfine analysis.

Experimental

Samples. Poly(oxyethylene) (10) hydrogenated castor oil (HCO) of the highest quality was donated by Nikko Chemicals Co. Ltd. (Tokyo, Japan) and used as received. The HCO had about 10 moles of oxyethylene moieties per mole of oil. The molecular structures of HCO and the spin probes used in this study are depicted in Fig. 1. The spin probes 7-(4,4-dimethyl-3-oxazolidinyl)-oxyoctadecanoic acid, so-called 7-doxylstearic acid (7-DSA), and the related compounds 12-doxylstearic acid (12-DSA) and 16-doxylstearic acid (16-DSA) were obtained from Aldrich Chemical Co. and used as received. All other reagents were of the highest grade obtainable and used as received.

Sample Preparation. Sample solutions were prepared as follows. A weighed amount of HCO was dissolved in a few milliliters of chloroform. The spin probe was dissolved in ~0.3 mL of chloroform and mixed with the HCO solution. After evaporation of the chloroform in a rotary evaporator, a 10 wt % dispersion of HCO/spin probe in distilled water (Wako Pure Chemical Ind. Ltd., Japan) was prepared. The test tube containing the dispersion solution was agitated on a vortex mixer until it was completely dispersed. The final concentration of the spin probe was approximate-

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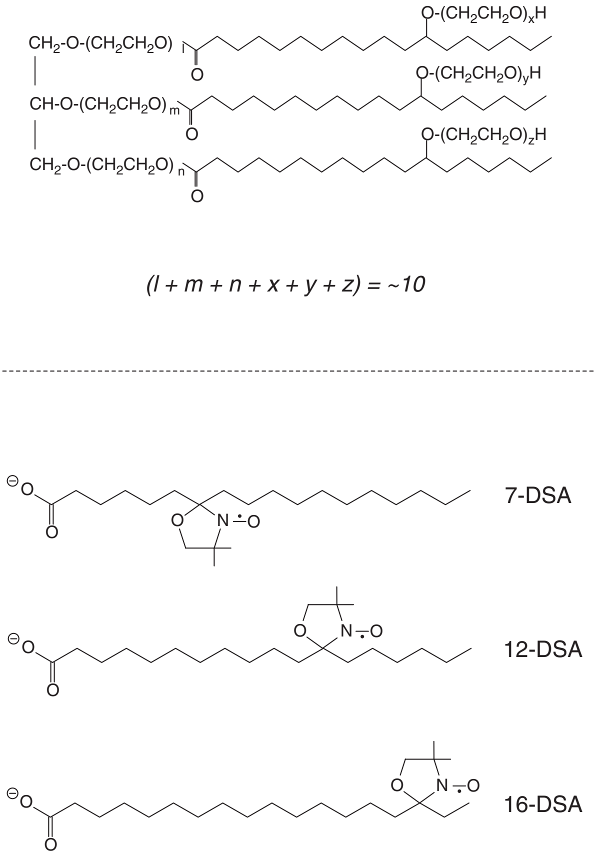

Fig. 1. The molecular structures of poly(oxyethylene) (10) hydrogenated castor oil (HCO) and various doxylstearic acids (DSA) used.

ly 100 μmol dm^{-3}.

Deoxygenation. For CW ESR, the sample solutions were deoxygenated for about 15 min in an AtmosBag (Aldrich) and the solutions were put into capillaries (I.D., 0.9 mm; O.D., 1.4 mm). Then, one end of the capillary was sealed. The sample capillary was inserted into a 3 mm ESR tube in the AtmosBag and taped around the tube cap. The detailed methods are also described elsewhere.[5]

CW EPR Measurements. ESR measurements were made with a 9 GHz JEOL FE 1XG spectrometer with a TE$_{011}$ cylindrical cavity. The sample temperature was controlled by nitrogen gas flow through the Dewar using a JEOL ES-DVT system. ESR signals were digitized using a Scientific Software Services data acquisition system (Illinois, USA). The microwave frequency was measured using an EMC-14 X-band microwave frequency counter (Echo Electronics Co., Ltd., Japan). The sample temperature was measured using a thermocouple (Omega HH11, USA) just above the cavity during the measurements. The detailed ESR settings are also described elsewhere.[5]

Calculation of the Slow-Tumbling Spin Probe. The slow-tumbling motions of the aliphatic spin probes were calculated using a computer program. The nonlinear least-squares fitting program called NLLS analyzes the ESR spectra based on the stochastic Liouville equation.[6,7] The latest version of the fitting program modified by Budil et al. was used.[8] The simulation of the ESR spectra for spin probes incorporated into multilamella vesicles was carried out using a microscopically ordered but macroscopically disordered (MOMD) model introduced by Meirovitch et al.[9] This model is based on the characteristics of the dynamic structure of lipid dispersions. For example, lipid molecules are preferentially oriented by the local structure of the bilayer, but the lipid bilayer fragments are overall distributed randomly. The spectrum from the dispersion sample can be regarded as a superposition of the spectrum from all of the fragments.

The lipid and DSA molecules in the membrane bilayer experience the ordering potentials, which restrict the amplitudes of the rotational motion. The orienting potential in a lipid bilayer, $U(\Omega)$, determines the orientational distribution of molecules with respect to the local ordering axis of the membrane bilayer. It can be expressed as an expansion in generalized spherical harmonics,[10,11]

$$-U/kT = c_0^2 D_{00}^2(\Omega) + c_2^2(D_{02}^2(\Omega) + D_{0-2}^2(\Omega)) + \dots, \quad (1)$$

where $\Omega = (\alpha, \beta, \gamma)$ are the Euler angles between the molecular frame of the rotational diffusion tensor and the local director frame. The $c_0{}^2$ and $c_2{}^2$ are dimensionless potential energy coefficients, k is the Boltzmann constant, and T is the absolute temperature.

The order parameter, S_0, is defined as

$$S_0 = \langle D_{00}^2 \rangle = \left\langle \frac{1}{2}(3\cos^2\gamma - 1) \right\rangle = \frac{\int d\Omega \exp(-U/kT) D_{00}^2}{\int d\Omega \exp(-U/kT)}, \quad (2)$$

which measures the angular extent of the rotational diffusion of the nitroxide moiety. Gamma (γ) is the angle between the rotational diffusion symmetry axis and the z-axis of the nitroxide axis system; z is the axis of the nitrogen 2p$_z$ atomic orbital, and x-axis is along the N–O bond. The local or microscopic ordering of the nitroxide spin probe in the membrane is characterized by the S_0 value. A larger S_0 value indicates very restricted motion. Therefore, S_0 reflects the local ordering of bilayer molecules in the disordered membrane dispersions.

The rotational diffusion of a doxylstearic acid spin probe is defined by the components of the rotational tensor, $R_\parallel$ and $R_\perp$, along axes parallel and perpendicular, respectively, to the symmetry axis of the rotational diffusion tensor. These parameters were determined by comparing the line shape of slow-motional experimental ESR spectra with the simulation. In a calculation of the experimental spectra, the principal components of the **A** and **g** tensors obtained by Ge et al. were used.[12] The best-fit **A** tensor components of the rigid limit spectra are listed along with the **g** tensors in Table 1. Further detailed descriptions are also given elsewhere.[5]

Results and Discussion

Figure 2 (left column) shows the representative ESR spectra of DSA/HCO in aqueous dispersions at 20 °C. The ESR spectrum of 7-DSA clearly shows parallel and perpendicular hyperfine components. The spectrum of 12-DSA also shows both components, but not as clearly as that of 7-DSA. On the other hand, the ESR spectrum of 16-DSA shows only three ^{14}N lines. However, not all three lines are of equal intensity. Especially,

Table 1. Principal Values of the Tensors Used in the Calculation of S and S_0 Values[12]

	A_{xx}	A_{yy}	A_{zz}	A_0
7-DSA	0.66	0.48	3.39	1.51
12-DSA	0.66	0.48	3.39	1.51
16-DSA	0.61	0.56	3.42	1.53

Unit is mT. $g_{xx} = 2.0086$, $g_{yy} = 2.0063$, and $g_{zz} = 2.0025$ for all three spin probes.

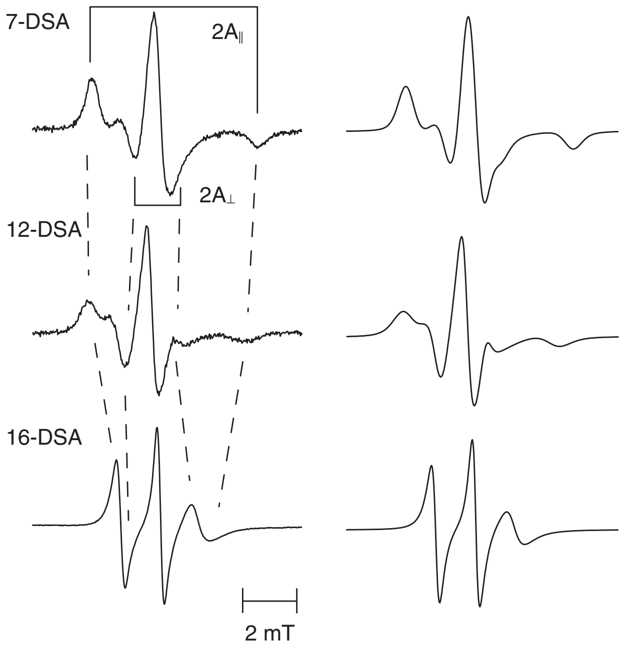

Fig. 2. Representative experimental (left column) and simulated (right column) ESR spectra of 7-DSA, 12-DSA, and 16-DSA observed in aqueous dispersions at 20 °C are shown. In the left column, parallel (2A$_\parallel$) and perpendicular (2A$_\perp$) hyperfine components are indicated as an example. The principal **A** and **g** tensor components are taken from the Ref. 12.

the high-field line intensity is very small. Based on the ESR spectra, the probe site of 7-DSA is more immobilized than those of 12-DSA and 16-DSA. The three-line spectrum of 16-DSA at the temperature range studied shows that the probe site is flexible. Thus, the ESR spectra show that the spin moiety of 7-DSA is more rigid and going further down the membrane chain, flexibility increases.

The degree of inclination for the principal axis of the nitroxide radical against the rotational axis of the long-chained probe molecule represents order–disorder molecular assemblies of the HCO membrane. The order parameter indicates the membrane chain dynamics and micro-viscosity of the medium in which the spin probe is incorporated. The conventional order parameter (*S*) is determined by the hyperfine coupling of ESR spectrum according to the following relations:[2]

$$S = \frac{A_\parallel - A_\perp}{A_{ZZ} - \frac{1}{2}(A_{XX} + A_{YY})} \cdot \frac{a}{a'}, \tag{3}$$

$$a' = \frac{A_\parallel + 2A_\perp}{3}, \tag{4}$$

where a is the isotropic hyperfine value; A_{XX}, A_{YY}, and A_{ZZ} are the principal values of the spin probe. The values of 2A$_\parallel$ and 2A$_\perp$ are obtained from the ESR spectrum. The experimental hyperfine couplings of 2A$_\parallel$ and 2A$_\perp$ are shown in Fig. 2 (left column). The order parameter indicates that the *S* value increases with the anisotropy of the probe site in the membrane. On the other hand, the *S* value becomes zero for completely isotropic rotation of the nitroxide radical. The order parameter also decreases when the temperature increases, because the micro-viscosity of the molecular assemblies decreases with the temperature rise.

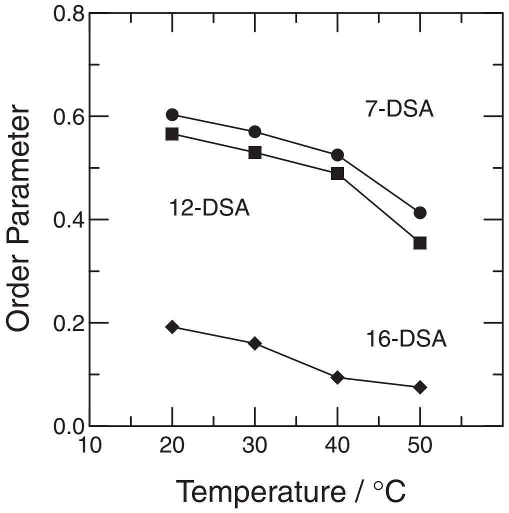

Fig. 3. Profile of the conventional order parameter (*S*) for various DSAs as a function of the temperature. Filled circles, filled squares, and filled diamonds represent 7-, 12-, and 16-DSA, respectively.

The order parameters (*S*) calculated using the hyperfine coupling values are presented in Fig. 3. The obtained *S* values for 7-, 12-, 16-DSA in the membrane at 20 °C were 0.60, 0.57, and 0.19, respectively. The *S* results show that 7-DSA and 12-DSA have similar values throughout the temperatures studied. Both values drop by about 30% from 20 to 50 °C. That is, the chain order for both probe moieties shows a similar temperature behavior. The *S* value of 16-DSA slightly decreases from 0.19 to 0.07 with an increase in the temperature. This value is much lower than that for 7-DSA and 12-DSA. This suggests that the spin site of 16-DSA is located in a flexible region. Although the observed ESR line shapes of 7-DSA and 12-DSA are very different, the *S* values for both are very similar throughout the temperatures studied.

The conventional order parameter as a function of the nitroxide position is shown in Fig. 4. The tendency is the same for the two different temperatures. The chain order is relatively high up to the 12th carbon, and then decreases rapidly toward the 16th carbon. The results show that the membrane flexibility increases significantly after the 12-position of the DSA probe moiety.

The conventional hyperfine analysis gives limited information concerning the probe environment at different positions in the HCO membrane. As pointed out above, changes in the ESR line shape do not always reflect the order parameter (*S*). This analysis cannot differentiate subtle spectral differences between 7-DSA and 12-DSA throughout the temperatures. Modern computational analysis of the ESR spectra provides new qualitative information regarding the membrane chain ordering. The simulation analysis of ESR line shape yields the order parameter (S_0), as described in the experimental section. The calculated spectra are presented along with the experimental spectra in Fig. 2 (right column). Satisfactory agreement between the experimental and calculated spectra was obtained. Thus, a spectral simulation will provide a qualitative S_0, which

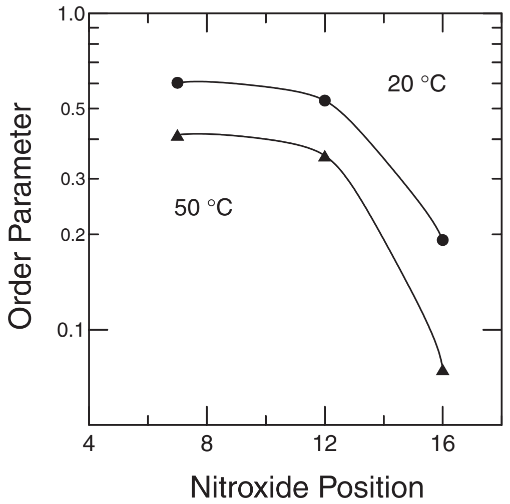

Fig. 4. The conventional order parameter (*S*) for the spin probes as a function of the nitroxide position at two different temperatures.

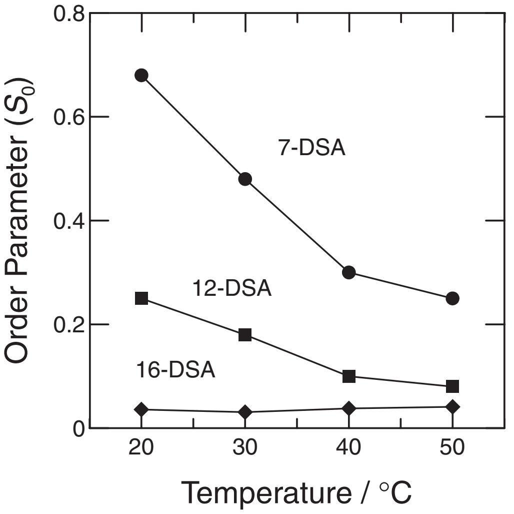

Fig. 5. Variation of the order parameter (S_0) for the spin probes in dispersions of HCO versus temperature obtained from the slow-motional ESR spectral simulation. Filled circles, filled squares, and filled diamonds represent 7-, 12-, and 16-DSA, respectively.

gives the microscopic chain ordering in the membrane.

The S_0 values obtained by the simulation are presented as a function of the temperature in Fig. 5. The S_0 values for 7-, 12-, and 16-DSA at 20 °C were 0.68, 0.25, and 0.04, respectively. The S_0 value for 16-DSA was much smaller than those of 7-DSA and 12-DSA, and did not change much throughout the temperatures studied. Thus, the S_0 together with *S* results suggest that the membrane chains of the bilayer portion of the membrane are well ordered in the region for 7-DSA and very fluid in the 16-DSA region. It is reasonable to infer from the previous order parameters[13] that the values of 0.6–0.7 and ~0.1 are the rigid and the fluid region in the membrane, respectively. The simulation results also suggest that the chain order of 12-DSA is in-between 7- and 16-DSA. The order parameter of 7-DSA is the most temperature sensitive from 20 to 40 °C. The temperature dependence and the order parameter of 12-DSA are quite different from those obtained by the hyperfine analysis.

Table 2. Parameters Obtained from the NLLS Calculation of ESR Spectra for Various Spin Probes in HCO Membrane at 20 °C

	S_0	$R_\perp/s^{-1}$	$R_\parallel/s^{-1}$
7-DSA	0.68	1.9×10^7	2.8×10^6
12-DSA	0.25	2.6×10^7	1.3×10^7
16-DSA	0.04	8.6×10^7	2.1×10^9

The magnetic parameters used in the simulations are the same as listed in the Table 1. Rotational correlation time (τ_R) can be estimated by $1/(6R_\perp)$.[3]

The recent investigation of the rotational diffusions of doxylstearic acid spin probes showed that not only the temperature behaviors, but also both probe motions, are different.[5] The two dynamic values, parallel ($R_\parallel$) and perpendicular ($R_\perp$) rotational diffusion coefficients, obtained by the simulation are given along with S_0 in Table 2. The $R_\perp$ and $R_\parallel$ values of 7-DSA at 20 °C are low, suggesting an environment that hinders the rotation along directions perpendicular and parallel to the long axis of the spin probe. The slower rotational dynamics yields relatively large S_0. The effective tilt angle can be estimated using Eq. 2. The effective tilt angle (27°) suggests that the rotational dynamic axis is nearly perpendicular to the membrane surface. On the other hand, the $R_\perp$ and $R_\parallel$ values of 12-DSA at 20 °C are faster compared with those for 7-DSA. Both values indicate a relatively rapid rotation. In addition, the S_0 value of 0.25 gives an effective tilt angle of 45°. These values for 12-DSA are attributable to the segmental rotational mobility of the membrane chain.

It is notable that the slow-tumbling simulation of 16-DSA has a greater calculation error than that of 7-DSA or 12-DSA. The expected error is normally a few percent, depending on the fitting.[11a] The calculation error for 16-DSA is two to three-times larger because of less chain ordering. However, the calculation errors do not affect the conclusions deduced from the parameters that correspond to the best fit.

Furthermore, the previous investigation of the spin-lattice relaxation time (T_{1e}) has shown that there is a noticeable difference in T_{1e} between 7-DSA and 12-DSA.[4,5] The T_{1e} values for 7- and 12-DSA at 21 °C were 9.1 and 5.5 μs, respectively. The S_0 values for 7- and 12-DSA are consistent with those for the relaxation times. It is important to point out that T_{1e} is sensitive to the overall molecular tumbling of the probe as well as the internal motion within the membrane chain. Thus, these results imply that each probe moiety has a different chain ordering.

Conclusions

The present ESR and the modern slow-tumbling simulation provide the qualitative membrane order. The ESR spectral patterns suggest that the tumbling motion of the nitroxide moiety of the probe increases as the position moves down the inner HCO membrane. The conventional analysis measuring $2A_\parallel$ and $2A_\perp$ gives limited information concerning the probe moiety in the membrane. The order parameter shows that 7- and

12-DSA have similar S values throughout the temperatures studied. However, the order parameters (S_0) obtained by a modern computational analysis are significantly different. The probe moiety of 12-DSA is relatively flexible. In addition, the chain ordering for 7- and 12-DSA decreases differently when the temperature increases. The S_0 results are consistent with the ESR spectral patterns as well as the previously obtained relaxation times.[5] Thus, the present results provide the detailed chain ordering and dynamics of the HCO membrane.

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