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DEPOLARIZED LIGHT-SCATTERING STUDIES OF BILAYER STRUCTURES IN PHOSPHOLIPID VESICLES

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Depolarized light scattering has been used to investigate the hydrocarbon chain packing of phospholipids in vesicles below the phase transition and ordering of their chains above the phase transition. The chain packing and ordering have been demonstrated for vesicles of L-α-dipalmitoylphosphatidylethanolamine and some phosphatidylcholines of different hydrocarbon chain lengths. Anisotropy ratios for phospholipid vesicles could be determined by measuring depolarization ratios for several vesicle sizes at low concentrations of the lipids. The following results were obtained. Hydrocarbon chains of L-α-dimyristoyl and distearoylphosphatidylcholines below their phase transitions pack at tilting angles in good agreement with X-ray diffraction data. On the other hand, hydrocarbon chains of dipalmitoylphosphatidylethanolamine pack perpendicular to the bilayer surface. Values of the averaged order parameter for dimyristoyl, dipalmitoyl and distearoylphosphatidylcholines at 2.5°C above their phase transitions are all the same and the value for dipalmitoylphosphatidylcholine is in agreement with results from ^{2}H-NMR experiments. The value of the order parameter for dipalmitoylphosphatidylethanolamine is slightly larger than that for dipalmitoylphosphatidylcholine.

Introduction

The structure and dynamic behavior of phospholipids in bilayer membranes have been investigated in detail using particularly X-ray diffraction [1–3], ^{2}H-NMR [4–7] and spin-label ESR spectroxopy [8,9]. X-ray diffraction is a useful method for studying the structures of phospholipids in bilayer membranes for gel-crystalline phases and ^{2}H-NMR spectroxopy for liquid-crystalline phases in general. In spin-label ESR spectroxopy, the hydrocarbon chains in a bilayer are distorted by the spin-label group in some cases [7]. On the other hand, the depolarized light-scattering method can be applied to both gel- and liquid-crystalline phases of lipid vesicles in water without any perturbations.

In the previous study, a general expression for the scattering light intensity based on the Rayleigh-Gans approximation [10] has been given for a hollow spherical shell with a thin wall in which anisotropic molecules are arranged radially on the surface [11]. The scattering angle dependence of the normalized scattering intensity, the so-called particle scattering factor $P(\theta)$, has been obtained by considering the interference of light scattered from different points of the hollow shell. The obtained expression of the scattering factor shows that partial scattering factors of P_{Hv} and P_{Vh} are zero at any scattering angles *

Abbreviations: DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine.

* It has been described in Ref. 11 that values of P_{Hv} and P_{Vh} are extremely small. But $P_{\mathrm{Hv}} = P_{\mathrm{Vh}} = 0$ exactly as pointed out (Yu, H., personal communication).

but P_{Hh} (90) is finite except in the case of the component molecules being isotropic, where H and V denote horizontally and vertically polarized scattered light, respectively, and h and v denote horizontally and vertically polarized incident light, respectively, in the scattering plane of incident and scattered beams. Furthermore, it has been shown that the depolarization ratio, ρ, at a scattering angle of 90°, defined by $(P_{Hv}(90) + P_{Hh}(90))/(P_{Vv}(90) + P_{Vh}(90))$, is extremely small for low values of the particle dimensions but increases rapidly with increasing particle dimensions and increasing anisotropy of the component molecules. This increase in ρ is due to an increase in P_{Hh} (90).

The previous study also showed experimentally that using DPPC vesicles, depolarized light scattering can be applied to study the chain packing of the lipid bilayer of vesicles in the gel phase: the anisotropy ratio for the vesicles was determined from the measured depolarization ratios. Then from the value of the anisotropy ratio the tilting angle of the chains was obtained.

The present work shows that the depoarized light-scattering method is a simple and rapid technique for studying bilayer structures of lipid vesicles in gel- and liquid-crystalline phases. The chain packing of lipids in vesicles below the phase transition temperature (T_c) and the ordering of their chains above T_c are reported for DPPE and some phosphatidylcholines of different hydrocarbon chain lengths.

Materials and Methods

Preparation of lipid vesicles

DPPE, DMPC, DSPC were purchased from Sigma Chemical Co. All the above phospholipids were used without further purification. Vesicles of these lipids were prepared by sonication with a bath-type sonicator (Branson, 220) for time periods. Weighed portions of the lipids were dissolved in chloroform. The solvent was removed on a rotary evaporator under vacuum at room temperature for about 5 h. The dried lipids were dispersed in triple-distilled water at a concentration of about 0.5 mg/ml. The dispersions were then sonicated at a temperature at least 10°C above T_c and passed through Millipore filters of either 450 or 220 nm pore diameter. In all the steps of preparation care was taken to avoid contamination.

Determination of vesicle size

The photon correlation method of light scattering (dynamic light scattering) was used to determine vesicle sizes. The incident light (Coherent Radiation, 4 mW unpolarized He-Ne laser) was focused on the center of a rectangular scattering cell. The scattered light was detected by photomultiplier (Hamamatsu T.V., R649S) after passing through two pinhole diaphragms. The photomultiplier was cooled to reduce thermal noise currents to 1–2 Hz. The photocurrent pulses were fed into a pulse counter and an autocorrelator. Hydrodynamic radii of the vesicles were determined from the data of the autocorrelator, assuming spherical shells for the shape of the vesicles. The standard equations of this method are given in Ref. 12.

Depolarized light-scattering measurements

Depolarization measurements were performed by means of a Glan-Thompson prism (extinction ratio $5 \cdot 10^{-5}$) placed in front of the two pinhole diaphragms. The depolarization ratio for water is not small (0.058) but its Rayleigh ratio (the intensity scattered per unit volume divided by the intensity of the incident light) is very small ($0.9 \cdot 10^{-6}$ cm^{-1}) [13]. Therefore, the light scattered by water could be neglected by means of the two pibholes. Depolarization for some liquids, viz., toluene, benzene and chloroform was measured and correct setting of the optical apparatus was checked by comparing the results with the reported values [13]. The scattering angle was set at 90°. The temperature of samples was controlled to within 0.5°C. Samples were not cooled below room temperature (18°C) in order to avoid condensation of water vapor on the outside of the scattering cell.

Results and Discussion

Table I shows the results of the observed depolarization ratio for monodisperse polystyrene spheres of 54.5 and 117 nm radii (Dow Chemical Co.) at low concentrations. The observed depolarization ratio, ρ', is the ratio of the observed photocurrents of horizontally ($I_{\parallel}$) and vertically $I_{\perp}$ polarized light scattered from the dispersion at a scattering angle of 90° for unpolarized incident light: $\rho' = I_{\parallel}/I_{\perp}$. Although polystyrene spheres might be optically isotropic solid

TABLE I

APPARENT AND EFFECTIVE DEPOLARIZATION RATIOS FOR POLYSTYRENE SPHERE DISPERSIONS

Radius (nm)	ρ' ($\times 10^{-2}$)	ρ ($\times 10^{-2}$)	Concentration (wt.%) ($\times 10^{-3}$)
54.5	0.22 ± 0.02	0	<5
117	0.63 ± 0.02	0.41 ± 0.04	<1.5

particles and the light scattered by water could be neglected, values of ρ' were not zero. According to the Mie theory [10], the horizontally polarized intensity of scattered light at 90° is finite for large isotropic spherical particles i.e., for large particle dimensions, R/λ and a large relative refractive index, $m = n/n_0$, where R is the radius of the particle, λ the incident wavelength in the medium, n the refractive index of the particle and n_0 that of the medium. However, since the value of m for dispersions of the polystyrene spheres is 1.188 at 20°C for wavelength of 589 nm and since R/λ for a polystyrene sphere of 54.5 nm radius is as small as 0.115, the intensity of horizontally polarized light scattered by 54.5 nm polystyrene spheres is extremely small: the ratio of the intensities of horizontally polarized scattering light (i_2) and of the vertical one (i_1), $i_2/i_1 = 0$ at 90°. Therefore, the finite value of ρ' for the dispersion of 54.5 nm polystyrene spheres may be considered as noise of the optical system. This noise might be caused by the following: dust in the path of incident and scattered beams, a few stains or defects on the face of optical glasses, slight condensation of water vapor on the light window of the cooled photomultiplier box (although double windows were used), and the difference in sensitivities of the photomultiplier to vertically and horizontally polarized beams. When I^s and I^n represent photocurrent pulses by particles of interest and by the noise, respectively, the observed depolarization ratio is written as $\rho' = (I^s_\parallel + I^n_\parallel)/(I^s_\perp + I^n_\perp)$. Since $I^s_\perp >> I^n_\perp$, the effective depolarization ratio, $\rho = I^s_\parallel/I^s_\perp$, is equal to $\rho' - (I^n_\parallel/I^s_\perp)$. Assuming polystyrene spheres to be optically isotropic, the value of ρ' for a dispersion of 54.5 nm polystyrene spheres can be set equal to the ratio of $I^n_\parallel/I^s_\perp$. It should be noted that this equality is valid only at low concentrations of the spheres. The apparent depolarization ratio was constant at low concentrations, but increased linearly with increasing concentration due to the multiple scattering effect. Since the extrapolated value of i_2/i_1 at 90° from the tables of Mie scattering of lower [14] is about $0.3 \cdot 10^{-2}$ for $2\pi R/\lambda = 1.5$, the effective depolarization ratio ρ for a 117 nm polystyrene sphere dispersion ($2\pi R/\lambda = 1.55$) may be a reasonable value (see also Table I).

Fig. 1 shows the results obtained of the effective depolarization ratio, ρ, for DPPE as a function of the vesicle size at temperatures of 30 and 68°C. Samples were in sufficiently low concentrations in order to avoid the multiple scattering effect. Solid lines in Fig. 1 show theoretical values for thin hollow spherical shells with different degrees of the anisotropy ratio, δ_h. In the case of a shell composed of cylindrical molecules, δ_h is defined by:

$$\delta_h = (\alpha_r - \alpha_p)/\bar{\alpha} \qquad (1)$$

$$\bar{\alpha} = (\alpha_\parallel + 2\alpha_\perp)/3$$

where α_r is the component of the polarizability tensor in the radial direction of the shell, α_p the component in a direction perpendicular to it, and $\alpha_\parallel$ and $\alpha_\perp$ represent the molecular polarizability along and

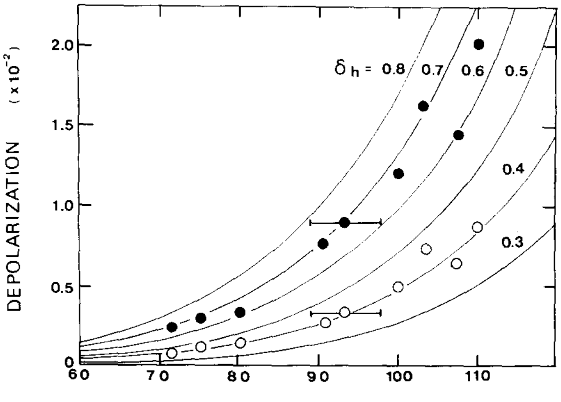

Fig. 1. Plots of the effective depolarization ratio vs. vesicle size for DPPE below and above the phase transition: ●, 30°C; ○, 68°C. Solid lines indicate theoretical values for thin hollow spherical shells with different degrees of the anisotropy ratio.

perpendicular to, respectively, the molecular axis. Chong and Colbow [15] reported the relative refractive index, m, to be 1.115 at 20°C for a wavelength of 589 nm for phosphatidylcholine dispersions. Substituting this value in the Rayleigh-Gans criterion [10], $(4\pi R/\lambda)|m-1|<<1$, the limitation of the particle dimensions is $R/\lambda<<0.7$. However, they proposed that the valid range of R/λ should be less than 0.2. In this case, the valid range of the lipid vesicle radius is less than 95 nm for the wavelength of light used in this work. In this range, values of the depolarization ratio for DPPE below T_c almost agree with the theoretical curve for an anisotropy ratio of 0.7.

The hydrocarbon chain packing of DPPE in the bilayers can be interpreted from this value of the anisotropy ratio as follows. If the lipid molecules pack at a tilting angle (ψ) to the radial direction of the shell, the anisotropy ratio of the shell can be written as [11]:

$$\delta_h = \delta_m(3\cos^2\psi - 1)/2 \qquad (2)$$

where δ_m is the anisotropy ratio of the a molecule and it is assumed that the molecules are oriented randomly in the shell but remain at the tilting angle and the shell is rapidly rotating during the time of the measurement. In a recent review of ^{31}P-NMR studies, Seelig [16] reported that conformations which are common to phosphoethanolamine and phosphocholine head groups are the *gauche* conformation of the O-C-C-N system and the *gauche-gauche* sequence of the phosphodiester linkage (C-O-P-O-C) both below and above T_c. Therefore, values of the anisotropy ratio for both head groups are probably smaller than those for the hydrocarbon chains. Then, in the case of the chain in the all-*trans* form, the anisotropy ratio of the molecule, δ_m, is equal to 0.711 using the values $\alpha_{\|} = 5.72$ and $\alpha_{\perp} = 2.96$ Å^{-3} for the polarizability of the *trans* segment of the chain calculated by Ohki and Fukuda [17]. Therefore, it is concluded that the chains of DPPE pack perpendicular to the bilayer surface, assuming the all-*trans* form of the chains.

Fig. 2 shows the results obtained of the effective depolarization ratio for DMPC as a function of vesicle size at temperatures of 20 and 27°C. Values of the depolarization ratio at 20°C almost agree with the theoretical curve for δ_h of 0.5 and the values at 27°C agree with the curve for δ_h of 0.3. It is interesting to note that measurements at 20°C should be taken as soon as possible. Practically, when DMPC samples were incubated at about 20°C for several tens of minutes, values of the anisotropy ratio for the samples decreased and vesicle sizes increased considerably. This behavior might be caused by fusion of smaller vesicles [18]. It is considered that the decrease in the anisotropy ratio is probably cause by the structures of larger vesicles not being of typical multilayer forms.

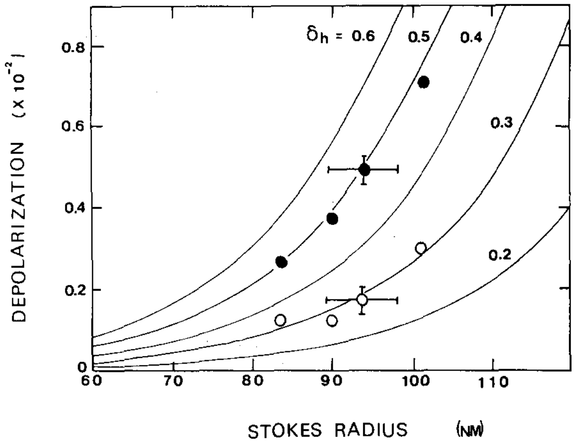

Fig. 2. Plots of the effective depolarization ratio vs. vesicle size for DMPC below and above phase transition: ●, 20C; ○, 27°C. Solid lines indicate theoretical values for thin hollow spherical shells with different degrees of the anisotropy ratio.

Fig. 3 also shows the results obtained of the effective depolarization ratio for DSPC as a function of vesicle size at temperatures of 30 and 58°C. Results of the anisotropy ratio and tilting angle for these vesicles of about 95 nm radius below T_c are summarized in Table II. The tilting angles for DMPC and DSPC can be compared with the results of X-ray studies [1,2] and are in good agreement, similarly to those for DPPC. Sheetz and Chan [19] have suggested the irregular packing of hydrocarbon chains in bilayers of small vesicles with large curvatures. Since the vesicles used in this work were so large, it is not necessary to consider this curvature effect. The results of the X-ray measurements made by Lagaly et al. [20] show some phases of kinked chains of 2*g*1 kinks (g^+tg^- or g^-tg^+ sequence) in bimolecular films of

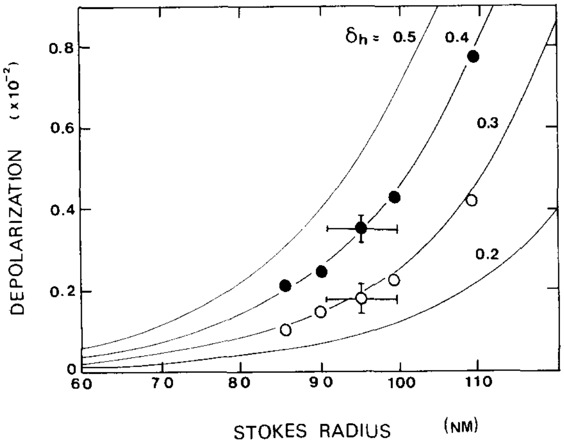

Fig. 3. Plots of the effective depolarization ratio vs. vesicle size for DSPC below and above phase transition: •, 30°C o, 58°C. Solid lines indicate theoretical values for thin hollow spherical shells with different degrees of the anisotropy ratio.

long-chain alkanols. Although chains of a lipid molecule have a few 2*g*1 kinks, the value of the anisotropy ratio may be not very different from that of the all-*trans* chains of the molecule. However, recent results of Raman [21,22] and density [23] studies suggest that below T_c there is substantial rotameric disorder in the *gauche* form. If so, the tilting angles may be overestimates, since the anisotropy ratio of the lipid molecule becomes smaller than 0.71. However, the estimate considering the *gauche* forms is difficult because only a few data are available about the gauche forms below T_c.

Anisotropy ratios for all lipid vesicles used here decreased markedly at T_c because their hydrocarbon chains entered more disordered states of *trans* and *gauche* conformations. ^{2}H-NMR experiments by Seelig et al. [7] show that in the disordered state, the long-chain axis of motional averaging is oriented perpendicular to the bilayer surface and so there is no collective tilt of the chain. Thus, it seems likely that ψ is equal to zero and decreases in the anisotropy ratio at the phase transition are only due to decreases in the anisotropy ratio of each molecule: $\delta_h = \delta_m$.

Neglecting the anisotropy of the head group, δ_m for a lipid molecule having *n* chain segments can be written as:

$$\delta_m = \frac{1}{n}\sum_{i=1}^{n}\delta_i(3\cos^2\beta_i - 1)/2 \qquad (3)$$

where δ_i is the anisotropy ratio of the *i*-th segment and β_i the angle between the bilayer normal and the direction of the *i*-th segment, which is defined as the normal on the plane spanned by the two C-H bond vectors. Unfortunately, polarizabilities for the *gauche* segment of the chain have not yet been reported but may be close to those for the *trans* segment. It is therefore assumed that the values δ_i are all equal to δ_t, the anisotropy ratio of the *trans* segment. Then $\delta_m = \delta_t\eta$, where η is termed the averaged order parameter of the molecule [24,25].

Table II also shows the results for the anisotropy ratio and the averaged order parameter, η, for vesicles of about 95 nm radius above T_c. The value of η for DPPC can be compared with the results obtained from ^{2}H-NMR experiments by Seelig and Seelig [4,5] (about 0.41 at 41°C and about 0.35 at 50°C) and is in agreement within experimental error but slightly

TABLE II

ANISOTROPY RATIOS (δ_h), TILTING ANGLES (ψ) AND ORDER PARAMETERS (η) FOR LIPID VESICLES

Data of the anisotropy ratio (δ_h) given for vesicles of about 95 nm radius. Phase transition temperatures determined by scattered light intensity measurements. δ_h: errors obtained by fitting the theoretical curves for thin hollow shells considering the uncertainty of the Stokes' radii and the depolarization ratios.

Phospholipid	T_c (°C)	δ_h below T_c	Temperature (°C)	ψ (degree)	δ_h above T_c	Temperature (°C)	η
DPPE	64.0	0.7 ± 0.1	30		0.43 ± 0.08	68	0.61 ± 0.11
DMPC	24.5	0.5 ± 0.1	20	26 ± 7	0.29 ± 0.06	27	0.41 ± 0.08
DPPC	41.5	0.5 ± 0.1 [a]	30	26 ± 7 [a]	0.30 ± 0.06 [a]	44	0.42 ± 0.08
DSPC	55.5	0.43 ± 0.08	30	31 ± 5	0.29 ± 0.06	58	0.41 ± 0.08

[a] Data from the previous study [11].

larger. This larger value of η should be caused by the polydispersity effect. Since the depolarization ratio grows sharply with increasing particle size (see solid lines in Fig. 1, 2 or 3), when vesicle size is distributed, the measured value of the depolarization ratio is affected considerably by larger vesicles. Samples used in this work were probably polydispersions.

It is of some interest to note that the three values of η for the phosphatidylcholines are all the same. Nagle and Wilkinson [23] reported, from density measurements, that the average change in the probability of a chain segment being *gauche* is the same value of about 0.2 for DMPC, DPPC and DSPC at T_c. Even if the number of *gauche* segments were not zero below T_c, the probability of a segment being *gauche* might be small for these lipids (0.08 for DPPC from Raman experiments [22]) and also might be not very different from each other. So it may be considered that the values of this probability above T_c are almost the same for these lipids. The same probability does not lead to the conclusion that the averaged order parameters are the same because *trans* segments of a chein can also be inclined to the bilayer normal due to *gauche* segments of the same chain. However, our results for η suggest that on the average, the chain configurations of DMPC, DPPC and DSPC are similar above T_c.

It is also interesting that the η value of DPPE is slightly larger than that of DPPC. Seelig [16] also reported in a recent review of ^{31}P-NMR experiments that the differences between the ethanolamine and choline head group conformations involve only the signs of the *gauche* conformations; the sign of the *gauche* conformation in the O-C-C-N system is the same as those of the *gauche-gauche* sequence of the phosphodiester linkage for phosphoethanolamine ($g^{\pm}g^{\pm}g^{\pm}$) but the signs are opposite for phosphocholine ($g^{\pm}g^{\mp}g^{\mp}$). It seems that the difference in signs of the *gauche* conformations of head groups cannot be the only cause of the difference in η between DPPC and DPPE. Therefore, the cause of the larger value of η for DPPE than that for DPPC is probably not only the differences in chemical structures and *gauche* conformations of the head groups but also the more ordered hydrocarbon chains in DPPE than in DPPC.

In conclusion, the results of depolarized light-scattering experiments show that: (1) below the phase transition, hydrocarbon chains of DPPE pack perpendicular to the bilayer surface and the chains of the three phosphatidylcholines (DMPC, DPPC and DSPC) pack at tilting angles in good agreement with X-ray diffraction data, and (2) above the phase transition, values of the averaged order parameter for the three phosphatidylcholines are all the same but the value for DPPE is slightly larger than that for DPPC. Thus, the depolarized light-scattering method is a simple and useful technique for studing the bilayer structures of lipid vesicles both below and above the phase transition. Much more accurate data on the bilayer structures of lipid vesicles will be obtained if a narrower distribution of the vesicle size, obtained by using gel filtration, and a higher accuracy in vesicle size determination are available.

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