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Optical birefringence of phosphatidylcholine liposomes in gel phases

Kiyoshi Mishima [a], Koichi Satoh [b] and Toshihiko Ogihara [c]

[a] *Department of Physics, College of Art & Science, Showa University, Fujiyoshida, Yamanashi 403,* [b] *Department of Physics, Premedical Course, Jikei University School of Medicine, Chofu, Tokyo 182 and* [c] *Department of Physiology, Faculty of Hygienic Technology, College of Environmental Health, Azabu University, Sagamihara, Kanagawa 229 (Japan)*

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A simple and rapid method for studying optical anisotropic properties of liposomes was proposed. Intensities of transmitted light through one spherical liposome of dipalmitoylphosphatidylcholine placed between two polarizers were measured at various wavelengths by a microscopic spectrophotometer. Large increases in the intensities were observed at the prephase-transition temperature, which were caused by an increase in the birefringence of the multilayer of the liposome. The birefringence values obtained from the intensity data were about 0.020 below the pretransition temperature and 0.028 above that temperature. These values are in good agreement with the results reported for the plane samples in which lipid bilayers are stacked. The obtained values of the birefringence were far lower than the values estimated from polarizabilities. This lower birefringence is attributed to disordering of the tilt direction in the multilayer. The degree of order of the liposome multilayers calculated from the birefringence increased by 38% at the pretransition. This simple method is applicable to the study of the multilayer structure of liposomes in water.

Introduction

The medical and technological applications of spherical liposomes have received recent attention. Optical properties of phospholipid dispersions have been studied by means of refractive index, scattered light intensity and turbidity measurements. Changes in the scattered light intensity and turbidity at the main phase transition were accounted for mainly by the change in the mean refractive index of the lipid lamellae [1,2]. Intensity changes in the scattered light from multilayered vesicles at the pretransition were also explained by the change in the refractive index [3]. A method of depolarized light scattering has been recently proposed and applied to phospholipid vesicles [4–7]. This method is based on the optical anisotropy of the hydrocarbon chains. These optical measurements for lipid dispersions are often affected significantly by the aggregation-disaggregation of lipid vesicle.

Optical microscopic observations for larger vesicles (liposomes) have been reported by some investigators. Harbich et al. [8] applied the phase-contrast microscopy method for the determination of the phase-transition temperatures of phosphatidylcholine liposomes. Yager et al. [9] observed changes in the shape and size of liposomes by the Nomarski interference method. Recently Petrov et al. [10] measured the intensity of the light transmitted through a phospholipid lamellar texture placed between crossed polarizers. The measured intensity of the transmitted light changes at pre- and main phase transitions, due to a change in the birefringence. Powers et al. [11] reported direct measurements of the birefringence in plane sam-

Correspondence: K. Mishima, Department of Physics, College of Art & Science, Showa University, Fujiyoshida, Yamanashi 403, Japan.

ples of lipid bilayer arrays with varying temperature and water content by use of a conoscopy method. In this method, however, the large sample (the diameter is about 1 cm) is required and the water content is limited.

We present here a new method to detect the birefringence in the liposome multilayers. A formula for the transmitted-light intensity through an anisotropic hollow sphere placed between two polarizers is given and applied to some phosphatidylcholine liposomes of different sizes. The intensity of the transmitted light through one spherical liposome was measured by use of a microscopy spectrophotometer. The birefringence in the multilayer was obtained from the transmitted-light intensity data. The degree of order for the multilayer was also obtained.

Theory of transmitted light through an anisotropic sphere

As is well known, the intensity of transmitted light through a plane parallel crystal plate between a polarizer and an analyzer is given by [12]:

$$I = A(\cos^2\chi - \sin 2\phi \sin 2(\phi - \chi) \sin^2(\delta/2)) \quad (1)$$

where A is the intensity of the incident light on the plate per unit area, χ the angle between the directions of vibrations passed by the polarizer and the analyzer, ϕ the angle between the direction of vibration passed by the polarizer and one of the two mutually orthogonal directions of vibrations in the crystal, and δ the phase difference between the two emerging beams. In the case of analyzer and polarizer perpendicular (crossed-Nicols or crossed polarizers), Eqn. 1 reduces to

$$I = A \sin^2 2\phi \sin^2(\delta/2) \quad (2)$$

Let n', n'' represent the refractive indices for the two emerging bemas, λ the wavelength of the incident light and ρ the mean geometrical path of the two beams. When the difference $n'' - n'$ is small compared with n' and n'', the phase difference, δ, is given by

$$\delta = (2\pi/\lambda)\rho(n'' - n') \quad (3)$$

and the difference, $n'' - n'$, for a uniaxial crystal plate is written

$$n'' - n' = (n_e - n_o) \sin^2\theta \quad (4)$$

where n_o and n_e are the ordinary and extraordinary refractive indices, and θ is the angle between the optical axis and the mean path of the two beams. As is well known, the difference $\Delta n = n_e - n_o$ is called the birefringence.

Let us consider a hollow sphere in which the shell is composed of uniaxial crystals of Δn and the optical axes of the crystals are radially oriented. To begin with, we will assume that $\Delta n \ll n_e$, n_o and so $n'' \approx n'$. The direction of two propagated beams in the shell always changes with the change of the optical axis. However, this change of the direction is negligible, if $n'' \approx n'$. Therefore, the mean geometrical path of the two beams, ρ, for the case of the propagation in the shell ((a) in Fig. 1)

$$\rho = 2R_o \cos\beta \quad (5)$$

and, for the case of the propagation in both the shell and the inside medium ((b) in Fig. 1)

$$\rho = 2(R_o \cos\beta - R_i \cos\beta'), \quad (6)$$

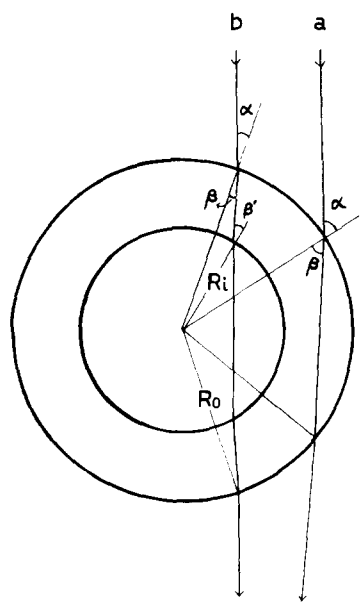

Fig. 1. Mean geometrical paths of ordinary and extraordinary beams propagated through (a) a shell, and (b) both the shell and the inside medium.

where R_o, R_i are the outer and the inner radius of the shell, and β, β' are the angles of refraction as shown in Fig. 1. In the limiting case of $\beta' = \pi/2$, obviously Eqn. 6 reduces to Eqn. 5. Next we will approximate the difference $n'' - n'$ as averaging over the light path for the factor $\sin^2\theta$. Then, for the case of (b)

$$n'' - n' = \Delta n \cdot \langle \sin^2\theta \rangle_{AV}$$

$$= \frac{\Delta n}{2}\left(1 - \frac{\sin 2\beta - \sin 2\beta'}{2(\beta - \beta')}\right) \tag{7}$$

The difference $n'' - n'$ for the case of (a) is replaced by Eqn. 7 with $\beta' = \pi/2$. It is noted that Δn is approximately equivalent to that of the same uniaxial crystal plate. This approximation is available for shells with large curvature. When the optical axes of the crystals are not radially oriented, Eqn. 7 is invalid, since β and β' are not angles between the optical axes and the light path. However, if the average directions of the optical axes are radial, Eqn. 7 is valid on average, where Δn is the average birefringence. Then the phase difference, δ, for the shell is written as

$$\delta = \frac{2\pi}{\lambda}\Delta n(R_o \cos\beta - R_i \cos\beta')\left(1 - \frac{\sin 2\beta - \sin 2\beta'}{2(\beta - \beta')}\right) \tag{8}$$

where $\beta' = \pi/2$ for case (a). When the phase difference is calculated geometrically from the light path difference of the two beams, exactly the same expression as Eqn. 8 is obtained.

We now consider the transmitted-light intensity through a shell illuminated by parallel incident light. The intensity of trnasmitted light under crossed-Nicols is the sum of Eqn. 2 over the shell

$$I = \int A \sin^2(2\phi) \sin^2(\delta/2)\, dS \tag{9}$$

where $A\,dS$ is the intensity of the incident light flux with the cross-sectional area of dS. When this incident light flux illuminates an area of the shell surface dS' with the angle of α, the area dS is described by

$$dS = dS' \cos\alpha = R_o^2\, d\phi \sin(2\alpha)\, d\alpha/2$$

Therefore, the intensity is

$$I = \int_0^{2\pi} (A/2) R_o^2 \sin(2\phi)\, d\phi \int_0^{\pi/2} \sin^2(\delta/2) \sin 2\alpha\, d\alpha \tag{10}$$

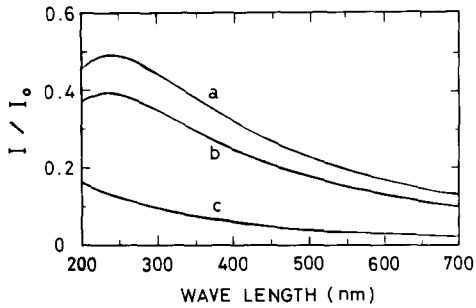

Fig. 2. Intensity of transmitted light through a shell, I/I_0 as a function of wavelength λ for different values of R_i/R_o: (a) $R_i/R_o = 0.1$; (b) $R_i/R_o = 0.4$; (c) $R_i/R_o = 0.7$. For all curves, the outside radius, $R = 5\ \mu m$, the birefringence, $\Delta n = 0.02$ and the relative refractive index of the shell to the medium, $m = 1.4$.

Since $I_0 = \pi A R_o^2$ is the total intensity of the illuminated light, the first integral of Eqn. 10 is equal to $I_0/2$. The second integral is divided into two terms of cases (a) and (b). Now we get the final formula of the transmitted-light intensity with crossed-Nicols as follows

$$I = \frac{I_0}{2}\left(\int_0^{\alpha_0} \sin(\delta/2) \sin 2\alpha\, d\alpha + \int_{\alpha_0}^{\pi/2} \sin^2(\delta/2) \sin 2\alpha\, d\alpha\right) \tag{11}$$

where α_0 is the incoming angle for the beam propagated on the interface of the inside medium and the shell. This angle is easily shown to be $\alpha_0 = \arcsin(mR_i/R_o)$, where m is the relative refractive index of the shell to the medium. In the second term of Eqn. 11, the phase difference, δ, is taken as Eqn. 8 with $\beta' = \pi/2$.

Fig. 2 shows the ratio I/I_0 as a function of λ for different values of R_i/R_o and Fig. 3 shows I/I_0 for different birefringence, (Δn) values. Calculations were made by use of the Simpson method. Since the intensity changes markedly with Δn, the birefringence is obtained from the intensity measurements.

Experimental

Materials and Methods

1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) was purchased from Sigma Chemical Co. and used without further purification. The dry

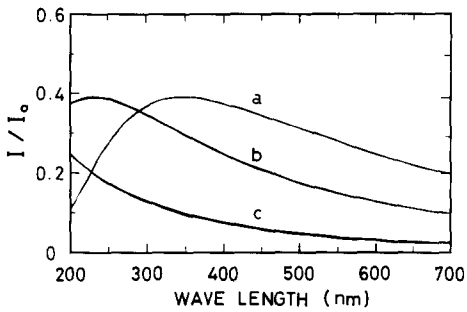

Fig. 3. Intensity of transmitted light through a shell, I/I_0 as a function of λ for different values of Δn: (a) $\Delta n = 0.03$; (b) $\Delta n = 0.02$; (c) $\Delta n = 0.01$. For all curves, $R_o = 5$ μm, $R_i = 2$ μm, $m = 1.4$.

lipid was dispersed in distilled water at a low concentration, about 0.5 mg/cm^3. The dispersions were incubated at about 50°C for about 1 h and then shaken gently. Some of them were sonicated for a few seconds with a bath-type ultrasonic apparatus. A drop of the dispersion was placed on a clean glass slide and covered with a cover glass. In some cases we used a 50 μm spacer. Wax was used as a seal to prevent evaporation. A smooth and spherical liposome was selected under optical microscopy.

An Olympus microscopic spectrophotometer system (MMSP-TU) was used in which the transmitted-light intensity could be measured through a micro-area of the sample. The center of the microbeam spot was put on the center of a selected liposome. The beam spot diameter was selected to be slightly larger than the liposome size in order to avoid the effects of other liposomes and dust. The light source for the spectrophotometer was a Xenon lamp. Since the intensity of the incident beam changed with wavelength, the intensity of transmitted light was normalized by the intensity of incident beam. Intensities of transmitted light through a 1/4 wave plate of 147.3 nm in retardation were measured under cross-Nicols with varying wavelength and correct setting of the optical apparatus was checked by comparison with the theoretical values. It was also shown that the absorption by slide glasses used and other optical apparatus was negligible in the wavelength range of 470–700 nm. Therefore, intensities of transmitted light through a liposome were measured with varying wavelength in that range. The intensity measurements were made for several liposomes of different size. The size of the liposomes was determined by microscopic measurements of their inner and outer diameters. The temperature of samples was controlled to within 1 Cdeg.

Results

Fig. 4 shows micrographs of dipalmitoylphosphatidylcholine liposomes and a micro beam spot, taken with natural light (a), and of the liposomes below (b) and above (c) the pretransition taken with a wavelength of 530 nm under cross-Nicols. In every liposome, the so-called dark-cross is observed. At the darkest regions in the ring, the polarization direction of the polarizer or analyzer is in the principal plane. The bright regions ex-

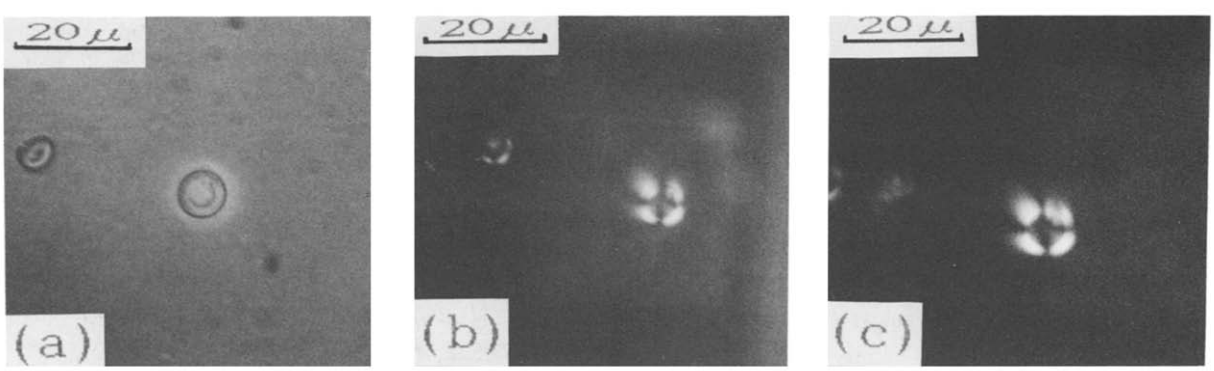

Fig. 4. Optical micrographs of dipalmitoylphosphatidylcholine liposomes observed with natural light below the pretransition (a), and with crossed-Nicols below (b) and above (c) the pretransition. In (a), A microbeamspot is also shown. The bright regions in (b) and (c) expand with haloes.

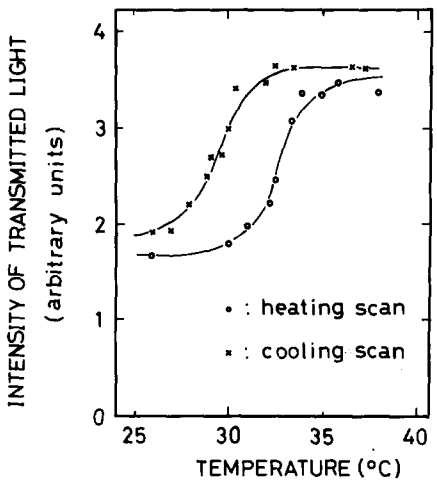

Fig. 5. Typical temperature dependence of the intensity of the transmitted light through a liposome of $R_o = 4.8$ μm and $R_i = 1.9$ μm illuminated at a wavelength of 470 nm.

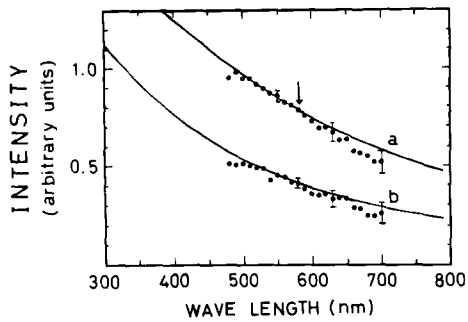

Fig. 6. Plots of transmitted-light intensities as a function of wavelength for a liposome of $R_o = 3.9$ μm and $R_i = 2.1$ μm at temperatures of (a) 37°C and (b) 25°C. Solid lines are the theoretical curves obtained as follows. The theoretical value of the intensity for the wavelength of 580 nm in vacuum was matched with the measured value at a temperature of 37°C for the same wavelength (marker). Then two curves for temperatures of (a) 37°C and (b) 25°C were obtained by fitting the measured points with varying birefringence, Δn. The value of Δn for best fitting is 0.019 for (a) and 0.028 for (b).

pand with haloes in the micrographs. The inner regions of the liposomes are dark because of the slight thickness and the optical axis parallel to the direction of incoming light. The intensities of transmitted light through one liposome under crossed-Nicols were detected by a photomultiplier. A typical temperature dependence of the intensity of the transmitted light is given in Fig. 5. Large increases in the transmitted-light intensity have been observed at the pretransition, while slight decreases of the scattered light intensity were observed [1–4]. The middle points of the intensity changes for heating scans were about 34°C in average. This value is in agreement with the pretransition temperature obtained by calorimetric measurements [13]. A substantial supercooling hysteresis was always observed in cooling down slowly enough. This behavior of the transmitted-light intensity in both heating and cooling scans are the same with the transmitted light through the lamellar textures [10]. Since no change was observed in the size of the liposome, this increase in the transmitted light intensity may be caused by the increase of the birefringence, Δn, of the liposome.

Fig. 6 shows plots of the transmitted-light intensities as a function of wavelength for temperatures above and below the pretransition. The intensities decrease with increasing wavelength because of the decrease in the phase difference δ. It is also noted that the intensities above the pretransition are about twice the intensities below the pretransition for wavelengths measured. Theoretical curves of the transmitted-light intensity calculated from Eqn. 11 are also shown in Fig. 6. The theoretical curves were obtained as follows. Although the refractive index of water, n_w, varies with the wavelength, the difference in n_w values was negligible in the calculation of the transmitted light intensity. We have used $n_w = 1.33$ for the wavelength of the incident light, $\lambda = \lambda_0/n_w$, where λ_0 is the wavelength in vacuo. The relative refractive index m in Eqn. 12 was used the value of 1.1 which was measured by Chong and Colbow [2]. For the inner and outer radius, the values measured from micrographs were used. Since the transmitted-light intensity data were measured relatively, the calculated value of the intensity for 580 nm of λ_0 was normalized with the values of the measured intensity for the same wavelength above the pretransition. Then two curves of the intensity above and below the pretransition were obtained by fitting the measured points to the calculated values with varying the birefringence. The birefringences of the liposome obtained by this fitting are 0.019 at 25°C and 0.028 at 37°C. In the same way, the birefringence of different sizes of liposome was obtained. The birefringences obtained were 0.028 ± 0.002 for the liposomes

above the pretransition and 0.020 ± 0.002 at the temperature range of 23–25°C. These values are in good agreement with the values measured by powers and Pershan [11] for the plane sample of lipid bilayer arrays with the water content of 21%.

Discussion

The birefringence of the lamellar phase of the liposomes was obtained from measurements of the transmitted light intensity with crossed-Nicols. Let us make an estimate of the birefringence value from polarizabilities for a uniformly ordered multilayer enclosing water between the lipid bilayers. Refractive indices n_e and n_o of a uniaxial liquid crystal are related with the principal polarizabilities α_e and α_o as follows [14]

$$\frac{n_e^2-1}{n_e^2+2}=\frac{4\pi}{3}\cdot\frac{N\alpha_e}{1+A_1\alpha_e} \tag{12}$$

$$\frac{n_o^2-1}{n_o^2+2}=\frac{4\pi}{3}\cdot\frac{N\alpha_o}{1+A_2\alpha_o} \tag{13}$$

where N is the number of molecules per unit volume and $A_1+2A_2=0$. A_1 and A_2 are constants by which the Clausius-Mosotti relationship can be adapted to the uniaxial crystal. When $n_e \approx n_o$, these adapted factors could be neglected and also $(n_e+1)/(n_e^2+2) \approx (n_o+1)/(n_o^2+2)$. Therefore, dividing Eqn. 12 by Eqn. 13, the birefringence, Δn, is obtained from the following equation

$$\Delta n=(\tilde{n}-1-\Delta n/3)\left(\frac{\alpha_e-\alpha_o}{\alpha_o}\right) \tag{14}$$

where $\tilde{n}$ is the average refractive index written by $\tilde{n}=(n_e+2n_o)/3$.

The polarizabilities for a C_2H_4 group in an alkane molecule have been obtained by Ohki and Fukuda [15] as $\alpha_{\parallel,C_2H_4}=2(\alpha_{CC}+2\alpha_{CH}+(2/3)\Gamma_{CC})$ and $\alpha_{\perp,C_2H_4}=2(\alpha_{CC}+2\alpha_{CH}-(1/3)\Gamma_{CC})$, where the indices $\parallel$ and $\perp$ stand for the principal direction of the molecule and a direction perpendicular to it, respectively. In the same way as Aragón and Pecora [7], we take numerical values of $\alpha_{CH}=0.65$ Å^3, $\alpha_{CC}=0.487$ Å^3 and $\Gamma_{CC}=0.54$ Å^3, and apply these values to the fully extended chains of a phospholipid molecule. We assume that the head group is optically isotropic. The head-group polarizability α_h can be obtained from the relation of $\alpha=3R/4\pi N_A$; here R is the molar refraction and N_A the Avogadro's number. We use the value reported by Aragon and Pecora [7] for the molar refraction of the head group ($R=65.9$ cm$^3\cdot$mol^{-1}), $\alpha_h=26.1$ Å^3. Furthermore, we consider the contribution of the water layer. The polarizability of a water molecule calculated from the molar refraction of $R_w=3.71$ cm$^3\cdot$mol^{-1} [16] is $\alpha_w=1.47$ Å^3. According to X-ray data, the cross-section of the head group is 42.8 Å^2–47.4 Å^2 [17] and the thickness of the water layer for dipalmitoylphosphatidylcholine is 17.2 Å at 37°C [18]. Thus the volume of the water layer per phospholipid molecule is 388 Å^3 ($45.1\times 17.2/2$) on average. Since the volume of a water molecule is about 30 Å^3, about 13 water molecules per lipid molecule are contained in the water layer. Then for a dipalmitoylphosphatidylcholine molecule with two fully extended chains and water molecules:

$$\frac{\alpha_{\parallel}-\alpha_{\perp}}{\alpha_{\perp}}=\frac{2\times 16\times\Gamma_{cc}}{(2\times 16(\alpha_{cc}+2\alpha_{ch}-\Gamma_{cc}/3)+\alpha_h+13\alpha_w)}$$

where we assume that the anisotropy is accounted for only by the chains. When lipid molecules are uniformly arrayed in the bilayers, $\alpha_e=\alpha_{\parallel}$ and $\alpha_o=\alpha_{\perp}$ should hold. Then we find that the birefringence for the plane multilayer is 0.079, using the value of 1.47 for the average refractive index at 35°C measured by Yi et al. [1].

The measured values of Δn for liposomes are far lower than the estimated value obtained above. X-ray diffraction analysis [18] shows that the lipid bilayers take the $L_{\beta'}$ phase below the pretransition and $P_{\beta'}$ phase between the pretransition and main transition. In both phases, the chains are fully extended and are tilted with respect to the normal to the bilayer plane. So the optical axes of bilayers are not radially oriented. In this case, as mentioned above, Δn is the average birefringence.

To begin with, we consider a case of uniform arrays of lipid molecules with same direction of the tilt throughout bilayers, and of uniform stacking of bilayers throughout the liposomes. In this case, since the optical axes are tilted equally with

respect to the radial direction of the liposome, β and β' in Eqn. 7 of the average for the factor $\sin^2\theta$ should be replaced by $\beta+\gamma$ and $\beta'+\gamma$, respectively, where γ is the tilting angle. Values of the tilting angle were reported by several authors [17–19]. Although we use the maximum value of γ ($=33°$) in the gel phase, which have been reported by Janiak et al. [18], the profile of the transmitted-light intensity with wavelength calculated is similar to that for the optical axes radially oriented, $r=0$. Therefore, for this case, there may be no large difference between the measured and estimated values of Δn. However, when lipid molecules in different bilayers are arrayed with different direction of the tilt preserving same tilting angle, the average birefringence becomes lower. Realistically, the multilayer may be composed of domains, in each which lipid molecules are arrayed with same direction of the tilt. The local disordering of lipid molecules also leads to a decrease of the average birefringence.

The degree of order defined by $S=(\alpha_e-\alpha_o)/(\alpha_{\parallel}-\alpha_{\perp})$ [14] can be calculated from the measured value of the birefringence as follows:

$$S=\left(\frac{\Delta n}{\bar{n}-1}\right)\Big/\left(\frac{\alpha_{\parallel}-\alpha_{\perp}}{\alpha_{\perp}}\right)$$

where we assume $\alpha_o \approx \alpha_{\perp}$ and neglect the factor $(\Delta n/3)$ in Eqn. 14. Using the average refractive index of 1.47, the degree of order for the multilayer is 0.24 at 25°C and 0.33 at 37°C. The increase in the degree of order at the pretransition is about 38%. Reported values of the tilting angle decrease slightly above the pretransition [18,19]. However, no significant increase in the average birefringence occurs for the first case of uniform arrays of lipid molecules with same direction of the tilt. Therefore, the increase of order may be attributed to more ordering in the tilt direction. This increase in ordering may be induced by bilayer rippling, which was observed in freeze-fracture electron micrographs [20,21].

In contrast to the slight decreases in scattered light and turbidity at the pretransition, the intensity of the transmitted light with crossed-Nicols increases considerably because of the increase in the birefringence. This change of the intensity is about twice that of the intensity below the pretransition. Therefore, the measurement of the transmitted-light intensity is useful for detection of the pretransition of liposomes. Furthermore, this is proposed as a simple method to detect the birefringence of liposomes. This method is an attractive technique for the study of the multilayer structure of liposome in water, since the samples are neither stained nor frozen. Also, this method is applicable for optically anisotropic tubes and rods of any curvature. In this work, the formula of the transmitted-light intensity was presented for a hollow sphere because of the simplicity of the calculation of the geometrical light path. A more realistic model for multilayer liposomes should consider multiple concentric hollow shells. Measurements of the transmitted-light intensity were carried out with varying wavelength. However, even if one uses monochromatic light as a light source, information about the multilayer structure of liposomes should be obtainable.

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