Probing Microscopic Orientation in Membranes by Linear Dichroism

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**ABSTRACT:** The cell membrane is an ordered environment, which anisotropically affects the structure and interactions of all of its molecules. Monitoring membrane orientation at a local level is rather challenging but could reward crucial information on protein conformation and interactions in the lipid bilayer. We monitored local lipid ordering changes upon varying the cholesterol concentration using polarized light spectroscopy and pyrene as a membrane probe. Pyrene, with a shape intermediate between a disc and a rod, can detect microscopic orientation variations at the level of its size. The global membrane orientation was determined using curcumin, a probe with nonoverlapping absorption relative to that of pyrene. While the macroscopic orientation of a liquid-phase bilayer decreases with increasing cholesterol concentration, the local orientation is improved. Pyrene is found to be sensitive to the local effects induced by cholesterol and temperature on the bilayer. Disentangling local and global orientation effects in membranes could provide new insights into functionally significant interactions of membrane proteins.

**Orientation of Liquid-Phase Bilayer:**

Our knowledge on biophysical properties of the lipid bilayer of a cell membrane is still rather limited. This is comprehensible considering the variety of lipids that constitute biological membranes and the complex chemical composition of the two leaflets. There is a delicate balance of weak hydrophobic, dispersive, and steric forces between molecules in the membrane, and their function is closely associated with the bilayer properties. Protein–protein interactions in the membrane are dependent on the local lipid environment, transbilayer asymmetry (lipid composition between the two leaflets), and lateral lipid asymmetry (domains). A fundamental property for a membrane probe to function is its orientation relative to the lipid bilayer. Detailed structural information can be obtained from protein crystals by X-ray crystallography, and so far a few hundred membrane protein structures have been resolved. Spectroscopic techniques such as polarized light spectroscopy, solid-state NMR, and fluorescence spectroscopy are used to gain information on the orientation of membrane proteins in native like lipid bilayers. The anisotropy of molecules in lipid bilayers is usually studied using macroscopically oriented membranes. Two contributions to anisotropy need to be considered: the macroscopic orientation of the membrane and the local microscopic orientation of molecules. The latter is a result of interactions between molecules and lipid acyl chains and/or polar headgroups. The separation of the two orientation contributions is rather difficult. Linear dichroism in combination with fluorescence anisotropy has been used to study the orientation of fluorophores embedded in lipid membranes to probe microscopic orientation effects in the lipid bilayers.

We propose the use of aromatic small molecules that have a shape that is an intermediate between disc and rod to gain information on the microscopic orientation and interactions at the level of the size of the molecules. Small planar molecules such as pyrene show variable orientation behavior with the order parameter of the short in-plane symmetry axis close to zero. With the idea that this parameter might be a sensitive indicator of local effects on the orientation distribution, we study the linear dichroism (LD) of pyrene in lipid bilayers. LD is defined as the difference in absorption of the light polarized parallel and polarized perpendicular relative to the flow direction. The difference between the absorbance with the two orthogonally polarized beams averages to zero if the sample is not aligned. The macroscopically oriented membranes used here are those of liposomes deformed into ellipsoids by laminar shear flow in a Couette cell. Linear dichroism (LD) reports on the orientation of the electronic transition moments. Pyrene has several distinct electronic transitions with nonoverlapping polarizations, which makes it easy to accurately probe its orientation using polarized light spectroscopy. Using curcumin, a probe that exhibits a nonoverlapping absorption relative to pyrene, and taking advantage of special environment-sensitive spectral properties of pyrene, we demonstrate that local and global effects of the orientation distribution in bilayers may be disentangled.

**EXPERIMENTAL SECTION**

**Preparation of Liposomes.** Liposomes were prepared by the lipid film hydration method. DOPC lipid and cholesterol (Avanti Polar Lipids, Inc.) in chloroform were mixed in the required molar ratios (1:0, 0.9:0.1, 0.8:0.2, and 0.6:0.4). The solvent was evaporated using a rotary evaporator. After at least 3 h under vacuum, the resultant dried lipid film was dispersed in 10 mM potassium phosphate buffer (pH 7.4) containing 50 wt % sucrose and allowed to hydrate for at least 1 h. Sucrose has the advantage of reducing the light scattering of the

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liposomes by matching their refractive index. The suspension was then extruded through polycarbonate filters with a pore diameter of 100 nm (at least 10 times) using a Lipex extruder (Northern Lipids Inc.). The final total lipid concentration was 1.3 mM in all samples. Stock solutions of pyrene and curcumin (Sigma-Aldrich) were prepared with ethanol (99.7%), and a specific volume was added to the liposomes in order to have final concentrations of, respectively, 25 and 4 μM (the total volume added was less than 0.5% of the total volume of liposomes).

**Linear Dichroism Spectroscopy.** Linear dichroism measurements were performed on a Chirascan CD spectrometer. All spectra were recorded between 200 and 550 nm in 1 nm increments at a scan speed of 120 nm/min and a bandwidth of 1 nm. The alignment of the liposomes was achieved by a custom-made outer-cylinder-rotation Couette flow cell with a path length of 1 mm (for the measurements at room temperature) or 0.5 mm for the temperature-dependence measurements (using a CS/CCA high-shear Couette cell accessory from Applied Photophysics Ltd.). The shear rate ranged from 80 to 3100 s⁻¹. No shifts or broadening in LD bands were seen with increasing shear rate, meaning that the improved macroscopic orientation was not associated with any significant site redistribution of chromophores. At least three data accumulations were made to generate an average for each measurement. Baselines at zero zero shear gradients were collected and subtracted from all spectra. The macroscopic orientation of the liposomes was probed using curcumin since its absorption band (λabs = 424 nm) does not overlap with the pyrene bands. We find curcumin to be a suitable membrane probe for liposomes, comparable to the standard probe retinoic acid. Note that when comparing the two probes they have opposite LD amplitudes because of their orthogonal preferred orientations relative to the membrane. Three intense transitions with pure polarizations (no overlapping of absorption bands) may be exploited for studying the orientation of pyrene: Lσ (with x polarization) at 337 nm, Bσ (with y polarization) at 273 nm, and Bπ (with z polarization) at 240 nm. Here we use the Lσ and Bπ transitions.

**RESULTS AND DISCUSSION**

The reduced LD (LD') is a dimensionless concentration- and path-length-independent variable defined as LD divided by the absorbance of the isotropic sample (Aiso(λ)). The geometry of the liposome experiment is as follows: the membrane normal D is aligned preferentially perpendicular to the flow direction, and the angles of the molecular orientation axes are defined relative to D (Figure 1). An uniaxial orientation distribution of the probe molecules around the membrane normal D may be assumed, which means that the probability of finding a given molecular axis at an angle θ from D is constant around a cone centered about D (Figure 1). Then the following relation between LD and membrane order parameters holds:

\[
LD'(λ) = \frac{LD(λ)}{A_{iso}(λ)} = 3S_D S_\alpha^\prime + S_\beta^\prime + S_\gamma^\prime + S_\delta^\prime + S_\varepsilon^\prime + S_\zeta^\prime
\]  

(1)

where S_D is a membrane orientation factor accounting for the degree of orientation of the membrane normal (D) in the laboratory system; e_α, e_β, and e_γ are the molar extinction coefficients for light polarized along the respective molecular axes x, y, and z; S_α, S_β, and S_γ are the order parameters for the orientation of the molecule relative to the membrane normal D and are defined as

\[
S_i = \frac{1}{2} (3 \cos^2 \theta - 1), \quad i = x, y, z
\]  

(2)

with θ being the angle between molecular axis i and membrane normal D. Since

\[
S_\alpha + S_\beta + S_\gamma = 0
\]  

(3)

only two molecular parameters are independent. Because of the measuring geometry with LD = A_2 – A_3, in which Z is a vector representing the flow direction and Y is a vector perpendicular to that direction and perpendicular to the propagation of light (Figure 1), the LD for a transition moment oriented parallel to the membrane normal D will exhibit negative values. Therefore, the global membrane orientation factor defined as

\[
S_D = \frac{1}{2} (3 \cos^2 \beta_D - 1)
\]  

(4)

with β_D being the angle between the flow direction and the membrane normal D, will be negative. In the case of a perfectly oriented lipid vesicle (elongated to an infinite cylindrical tube), β_D = 90° and S_D = 0.5. Note that this definition differs from that used in our previous formulas, where a perfect membrane orientation corresponded to S = 1. In that case, instead of the membrane normal, it is the long axis of the deformed liposome that was used as the reference direction (an infinitely elongated liposome aligns perfectly parallel to the flow direction). The orientation factors for the two notations relate as S = −2S_D.

The reason that we change to a new notation is the desire to have the membrane normal as the main reference axis and molecular orientation parameters to be in agreement with the general nomenclature for uniaxial systems, including membrane lipid bilayers.

Here, S_D is obtained from the curcumin LD signal at 424 nm:

\[
LD'(424 nm) = \left(\frac{3}{2}\right)S_D(3 \cos^2 90° - 1)
\]  

(5)

**Equation 1** may then be used to determine the ratio

\[
\frac{LD'(273 nm)}{LD'(337 nm)} = \frac{S_\beta}{S_\gamma}
\]  

(6)

and the separate order parameters

\[
LD'(273 nm) = \frac{LD(273 nm)}{A_{iso}(273 nm)} = 3S_D S_\beta
\]  

(7)

\[
LD'(337 nm) = \frac{LD(337 nm)}{A_{iso}(337 nm)} = 3S_D S_\gamma
\]  

(8)

Because of the high accuracy of measuring the ratio LD'(273 nm)/LD'(337 nm), eq 6 allows a higher precision for comparing relative changes between the two order parameters. For absolute size, eqs 7 and 8 have been used, after scaling with

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Figure 1. Orientation reference systems. Right: molecular orientations (characterized by parameters S_D) relative to a local axis and D, the membrane normal. The average orientation of D, related to the overall orientation of the deformed liposome (pictured as a cylinder on the left) is determined by the parameter S_D.
In this way, a physical interpretation of how the microscopic orientations are affected by the membrane properties can be obtained.

We measured the flow LD spectra of pyrene and curcumin incorporated into DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) liposomes prepared with 0–40 mol % cholesterol. Curcumin in DOPC liposomes shows a positive LD band at 424 nm, indicating that the transition moments at this wavelength are oriented perpendicular to the membrane normal. Previous linear dichroism studies show that the transition moments for the absorption band above 400 nm of curcumin are oriented along the long molecular axis of the molecule. Hence, the long axis of curcumin is oriented perpendicular to the membrane normal under the conditions studied here (curcumin/DOPC = 0.003). The location of curcumin in DOPC bilayers depends on the curcumin/lipid ratios. At ratios of up to 0.032, curcumin is located at the surface of the bilayer, whereas at higher ratios, the molecule intercalates into the membrane.

Pyrene shows negative LD peaks at 337 and 240 nm consistent with a preferred orientation of its longest in-plane symmetry axis (z) parallel to the lipid bilayer chains (Figure 2).

Because pyrene is quite sensitive to its environment, it was judged meaningful to carefully compare the absorption and LD spectra (Figure 2B). The LD spectrum of the L$_{a}$ transition shows a characteristic sharp vibrational structure indicating that all aligned pyrene molecules are in a nonpolar environment, consistent with a location close to hydrocarbon chains inside the lipid membrane. As for the short in-plane symmetry axis (y) given by the LD at 273 nm, the signal is positive in the more well-ordered system containing 20 and 40 mol % cholesterol but appears as a positive and as a negative peak, split in wavelength with the positive peak at slightly shorter and the negative at longer wavelength in samples with 0 and 10 mol % cholesterol (inset in Figure 2A). This behavior is similar to what has been observed in other contexts, in lamellar lipid bilayers as well as in stretched sheets of polyethylene. A small LD signal indicates a variable orientation, with the plane of the molecule imparting to the y axis a certain prevalence for being aligned parallel to the lipid chains, while any orientation of the z axis parallel to the lipid chains will, of course, make y perpendicularly oriented. When comparing the reduced linear dichroism (LD') of the pyrene long axis (337 nm) with the values for curcumin at 424 nm, a virtually linear relationship is observed as the shear force increases for the samples with 0, 10, and 20% cholesterol (Figure 3). For the sample with 40% cholesterol, it is the LD' at 273 nm versus that of curcumin at 424 nm that shows linear behavior (Figure 3). The linearity between the LD' at 273 or 337 nm and the LD' at 424 nm is an indication that the two probes can monitor the orientation of similar parts of the bilayer, which is a prerequisite for using eqs 7 and 8.

The orientation parameter $S_{D}$ slightly improves (in absolute value) for DOPC liposomes containing 10 and 20 mol % cholesterol compared to the sample without cholesterol, whereas the liposomes with 40% cholesterol are the poorest aligned, showing the lowest absolute values of $S_{D}$ (Figure 4).

The first strong transition ($L_{a}$) of pyrene, with a vibrational structure between 300 and 340 nm, has a pure polarization

![Figure 2. LD spectra at a shear rate of 3100 s$^{-1}$ (A) and absorbance spectra (B) of pyrene and curcumin in lipid bilayers of DOPC liposomes with 0 (black curve), 10 (gray), 20 (blue), and 40 mol % (purple) cholesterol (chol). Inset in A: Close-up view of the LD band at 270–280 nm. Inset in B: Short and long axis polarized transition moments of pyrene.](image)

![Figure 3. LD' of pyrene at 337 (top graph) and at 273 nm (bottom graph) plotted versus the LD' of curcumin at 424 nm for DOPC/cholesterol liposomes. The cholesterol (chol) contents are 0, 10, 20, and 40 mol %.](image)
Figure 4. Orientation factor $S_{zz}$ of DOPC/cholesterol liposomes as a function of the shear rate. The cholesterol (chol) contents are 0, 10, 20, and 40 mol %. $S_D$ values are determined using the LD$^e$ values of curcumin at 424 nm.

along the long axis ($z$ axis) and hence directly provides a value of $S_{zz}$ according to eq 1. The second strong transition ($B_b$), with an intensity similar to that of the first transition and with pure polarization along the in-plane short axis ($y$), shows a sharp absorption peak at 273 nm. As seen from Figure 2A, however, the corresponding LD is close to zero, indicating that $S_{yy}$ is near zero. In the presence of cholesterol, the tendency to align the $z$ axis parallel to the lipid chains increases as seen from increasing $S_{zz}$ (Figure 5). As the $z$ axis becomes better aligned, the $y$ axis adopts a more perpendicular orientation as expected. The near-zero LD for the $y$ axis of pyrene and corresponding near-zero $S_{yy}$ exhibit several interesting properties. The split LD peak at 273 nm indicates a distribution between slightly different environments and orientations that, to speak statistically balance each other: molecules with negative LD that are red-shifted by a few nanometers and others with positive LD that absorb a few nanometers to the blue compared to the center of the absorption maximum at 273 nm. The S-shaped LD is thus an effect of an inhomogeneous broadening whose microscopic distribution is revealed thanks to the fact that the infinitesimal spectral shifts are coupled to different orientations. In conclusion, the occurrence of two peaks is probably only the result of an overlap of a large number of LD spectra with different signs but with slight energy shifts relative to each other. The negative LD signal at around 273 nm is consistent with a $B_b$ ($y$-polarized) transition moment more parallel to the lipid chains (whose direction has the largest polarizability), whereas the positive band is consistent with a $B_a$ transition moment perpendicular to the lipid chains (less interaction with the transition, less red-shifted). That both kinds of orientation occur can be seen as an effect of nearly dislikelike behavior favoring both orientations of the in-plane short axis as well as the long axis parallel to the membrane normal $D$.

The split LD at 273 nm may tell something about the orientation distribution: the negative LD can be seen as corresponding to a subfraction of the molecular ensemble having positive $S_{yy}$ and the positive LD corresponding to a fraction with negative $S_{yy}$ values. In other words, the $S_{yy}$ value being close to zero is consistent with a broad distribution dictated both by the wish of the plane to be parallel to the membrane normal (dislikelike orientation) and the long axis to have the same orientation (rodlke orientation). Similar nearly dislikelike behavior with respect to the orientation direction of alkyl chains is also observed for pyrene when solubilized in a stretched polyethylene matrix.24 The ambiguous disk/rodl behavior is markedly changed by the presence of cholesterol. $S_{yy}$ decreases to more negative values while $S_{zz}$ increases. This is indeed the expected behavior for a better-oriented system because an increased $S_{zz}$ will statistically make the $y$ axis more perpendicular and thus $S_{yy}$ more negative.

The approximately 3-fold increment of $S_{zz}$ after the addition of 40% cholesterol to DOPC agrees well with the values of an average orientational order parameter that takes into account the chain tilt angle obtained for DOPC using wide-angle X-ray scattering.24 Our results are also in agreement with MD simulations of pyrene in a POPC membrane.25 That study shows that the $y$ axis of pyrene in POPC bilayers has a rather broad orientation distribution relative to the normal to the membrane plane, whereas the long axis shows a clear preference perpendicular to the membrane normal. With increasing cholesterol concentration, this distribution is predicted to sharpen up further. Such behavior is expected if repulsive forces by steric crowding (rigid steroid skeleton) would further force the plane of the molecule to align parallel to the lipid chains and is consistent with cholesterol playing an important role in ordering the lipid acyl chains. An important observation of the MD simulation study is that the effect of pyrene insertion in the bilayer is very small (the simulations were carried out with 150 lipid molecules and 2 or 4 pyrene molecules).25

Cholesterol depletion in cells was found to disrupt the orientation of a fluorophore located within the hydrophobic region of the membrane but not that of a surface-bound probe.9 Both dispersive and steric forces will favor an orientation of the longest dimension of pyrene parallel to the lipid chain direction as a result of the maximum polarizability and the maximum lever length, respectively. While the attractive forces may to a certain extent allow the presence of orientations of the normal to the aromatic molecular plane to be parallel to the lipid chain direction, the steric forces are anticipated to effectively suppress such orientations. The effect of such a steric exclusion is to increase the absolute values of the orientation factors for the pyrene long axis ($S_{xx}$) and short axis ($S_{yy}$).

Figure 5. Microscopic order parameters $S_{yy}$ and $S_{zz}$ of pyrene in DOPC/cholesterol liposomes plotted versus each other. $S_{yy}$ and $S_{zz}$ are obtained from the LD$^e$ values of pyrene at, respectively, 273 and 337 nm. The cholesterol (chol) concentration varies from 0 to 40 mol %. The liposomes were subjected to shear rates of between 320 and 3100 s$^{-1}$.

LD of DOPC liposomes with and without cholesterol was measured between 22 and 60 °C to determine whether our method is sensitive to the effects of temperature on the bilayer
structure. We observe that the absolute values of the global membrane order parameter \( S_y \) of DOPC and DOPC/cholesterol 0.6:0.4 liposomes slightly decrease on heating and increase on cooling (Figure 6). The \( S_{xy} \) and \( S_{zz} \) parameters of DOPC liposomes are only slightly affected by the temperature increase, although they are closer to zero. On cooling, \( S_{zz} \) goes back to the initial value but \( S_y \) remains almost zero (Figure 6). DOPC/cholesterol 0.6:0.4 shows larger \( S_y \) values and the \( S_{xy} \) values become more negative with the temperature increase. On cooling, \( S_{zz} \) reaches the maximum value and \( S_y \) has approximately the same initial value.

The lower orientation of the liposomes at higher temperatures can be explained by a decrease in viscosity of the sucrose buffer.

Figure 6. (A) Global membrane parameter \( S_y \) obtained from the LD' of curcumin at 424 nm for DOPC and DOPC/cholesterol 0.6:0.4 liposomes as a function of temperature. (B) Molecular order parameters \( S_{xx} \) and \( S_{yy} \) of pyrene in lipid bilayers of DOPC and DOPC/cholesterol 0.6:0.4 liposomes. The shear rate is 3100 s\(^{-1}\).

The less the viscous drag force predominates, the less deformed liposomes will be. \( S_y \) and \( S_{zz} \) give more insight because they have been corrected for in eq 1. For DOPC liposomes, it is not surprising that the order parameters of pyrene tend to zero with the temperature. It is well known that as the temperature increases, the DOPC bilayer becomes thinner and the area per lipid increases, reflecting the increase in the tilting of DOPC lipids. These changes are attributed to the increase in entropy with lipid chain rotational disorder, the increase in intermolecular entropy, and the increase in lipid headgroup hydration. We do not expect phase transitions to occur at the temperatures studied. X-ray diffraction analysis confirmed that DOPC alone forms only lamellar structures at temperatures of up to 100 °C. The sample containing DOPC and 0.4 mole fraction of cholesterol exhibits different behavior. When the temperature increases, the order parameters of pyrene improve. Although this effect may seem puzzling at first, it probably reflects the polymorphic behavior of the bilayer. Previous \(^{14}\)P NMR studies revealed that DOPC and cholesterol at a molar fraction of 0.4 show an isotropic pattern in addition to the bilayer pattern as the temperature increases. Lipid polymorphism was accompanied by the phase separation of cholesterol crystalline domains. Curiously, mixtures of DOPC with cholesterol behave differently with temperature when compared to other unsaturated phospholipids such as SOPC or DOPS. A plausible cause for better oriented \( z \) and \( y \) axes of pyrene in DOPC/cholesterol mixtures at high temperatures is the distribution of pyrene molecules within the different lipid phases. If a significant number of pyrene molecules are located in the ordered cholesterol phase, then this will translate into a better-oriented system. Molecular dynamics simulations indicate that the center of mass of pyrene lies at a depth similar of that of a cholesterol ring. Lipid-phase separation might result in a redistribution of pyrene molecules, which could accumulate in the cholesterol domains. On cooling, the samples partially retain the changes in microscopic orientation undergone with the temperature increase. The macroscopic orientation improves after the heating–cooling cycle. It has been described that vesicles become less rigid with the increased temperature in laminar flow. Although the effect was found to be reversible in water, this might not be the case in highly viscous environments such as in the presence of 50 w/w% sucrose.

What has been learned from this study may be summarized as follows. Small chromophores, such as pyrene that have a shape in between disc-shaped and rod-shaped and whose absorption peaks are shifted depending on the closeness and orientation relative to surrounding anisotropic solvent molecules, are useful for probing microscopic orientation effects in bilayers on the scale of the size of the probe molecule. We use pyrene, but what has been learned should be general and also applicable to other probe molecules. The order parameters \( S_{xx}, S_{yy} \), and \( S_{zz} \) describing the orientations of the principal axes of a solute probe molecule relative to the local membrane normal can be determined according to eq 1 once the global membrane orientation factor \( S_D \) can be independently determined, e.g., using an internal probe (eq 4). The value of the intermediate order parameter \( S_y \) (in-plane short axis) being close to zero reflects a broad orientation distribution. Combined with inhomogeneous spectral variations, LD may be used to diagnose microscopic orientation effects; for example, a positive \( S_y \) value and red-shifted LD for pyrene is consistent with a preferred orientation of the \( y \) axis parallel to lipid chains (stronger interaction). Correspondingly, a negative \( S_y \) with a smaller red shift is consistent with a greater long-axis (rodlike) orientation and less interaction of the \( y \) axis with lipid chains.

There are many situations when more information about the microscopic orientation behavior in membranes could be useful. It would be valuable to have probes that are specifically designed to monitor the orientation in certain lipid domains, such as in a cholesterol-rich domain or in the lipid cluster surrounding a membrane channel protein. In the future, we intend to study how the presence of proteins and other macromolecules may affect the local membrane orientation using pyrene and other probes.

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■ ABBREVIATIONS

LD, linear dichroism; LD', reduced linear dichroism; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; SOPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; SOPS, 1-stearoyl-2-
oleoyl-sn-glycero-3-phosphatidylcholine; DOPS, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine

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