Probing Microscopic Interactions in Membranes

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ABSTRACT: The cell membrane is an ordered environment, which anisotropically affects the structure and interactions of all its molecules. To monitor 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 cholesterol concentration using polarized light spectroscopy and pyrene as a membrane probe. Pyrene, with a shape intermediate between disc and rod, can detect microscopic orientation variations at the level of its size. The probe is found sensitive to the stiffening of a liquid phase bilayer induced by cholesterol. While the macroscopic orientation of the bilayer impairs with increasing cholesterol concentration, the local orientation is improved. Disentangling local and global orientation effects in membranes could provide new insights on functionally significant interactions of membrane proteins.

Our comprehensive knowledge on biophysical properties of the lipid bilayer of a cell membrane is still rather limited.[1] This is understandable 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 to its properties. Protein-protein interactions in the membrane are dependent on the local lipid environment, trans-bilayer asymmetry (lipid composition between the two leaflets) and lateral lipid asymmetry (domains).[2] A fundamental property for a membrane protein to function is its orientation relative to the lipid bilayer.[3] So far few hundred of membrane protein structures have been resolved but still their alignment in the membrane is not known.

The anisotropy of molecules in lipid bilayers is usually studied using macroscopically oriented membranes.[4-8] Two contributions need to be considered here, the macroscopic orientation of the membrane and the microscopic orientation of the molecule, which results from its interactions with lipid acyl chains and/or polar head groups. Their separation is rather difficult.[9] 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 as pyrene show ambivalent 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 exploit the linear dichroism (LD) of pyrene in lipid bilayers. Pyrene has several distinct electronic transitions with non-overlapping polarizations,[10] which makes it easy to accurately probe its orientation using polarized light spectroscopy. Using a membrane-surface probe, curcumin, which exhibits a non-overlapping absorption relative to pyrene, and taking advantage of special environment-sensitive spectral properties of pyrene, we demonstrate that local and global effects of orientation distribution in the lipid bilayer may be disentangled. Assuming an uniaxial orientation distribution of the probe molecules around the membrane normal (D), the following relation between LD, defined as the absorbance with light polarized parallel minus the absorbance

polarized perpendicular to flow direction, and membrane order parameters holds:

$$LD^r(\lambda) = \frac{LD(\lambda)}{A_{iso}(\lambda)} = 3\,S_D\,\frac{S_{xx}\varepsilon_x + S_{yy}\varepsilon_y + S_{zz}\varepsilon_z}{\varepsilon_x + \varepsilon_y + \varepsilon_z}, \qquad (1)$$

where S_D is a membrane orientation factor accounting for the degree of orientation of the membrane normal (D) in the laboratory system; ε_x, ε_y and ε_z are the molar extinction coefficients for light polarized along the respective molecular axes x, y and z; S_{xx}, S_{yy} and S_{zz} are the microscopic order parameters for the orientation of the probe relative to the membrane normal and are defined as

$$S_{ii} = (1/2)\,(3\,\langle\cos^2 D_i\rangle - 1), \quad i = x, y, z, \qquad (2)$$

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

$$S_{xx} + S_{yy} + S_{zz} = 0, \qquad (3)$$

only two parameters are independent. Due to the measuring geometry with $LD = A_Z - A_Y$, Z being a vector representing the flow direction and Y a vector perpendicular to that direction and perpendicular to the propagation of light, the LD for a transition moment oriented parallel with the membrane normal D will exhibit negative values. Therefore, the global orientation factor defined as

$$S_D = (1/2)\,(3\,\langle\cos^2 Z_D\rangle - 1), \qquad (4)$$

with Z_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) $Z_D = 90°$ and $S_D = -0.5$. Note that this definition differs from that used in our previous formulas, where a perfect membrane orientation corresponded to $S = 1$ (the relation is $S = -2\,S_D$).[11] Here, S_D is obtained from the curcumin LD signal at 424 nm:

$$LD^r(424\,nm) = (3/2)\,S_D(3\cos^2 90° - 1). \qquad (5)$$

Eq. (1) may then be used to determine the ratio

$$\frac{LD^r(273\,nm)}{LD^r(337\,nm)} = \frac{S_{yy}}{S_{zz}} \qquad (6)$$

and the separate order parameters:

$$LD^r(273\,nm) = \frac{LD(273\,nm)}{A_{iso}(273\,nm)} = 3S_D\,S_{yy} \qquad (7)$$

$$LD^r(337\,nm) = \frac{LD\,(337\,nm)}{A_{iso}(337\,nm)} = 3S_D\,S_{zz} \qquad (8)$$

Due to the high accuracy of measuring the ratio $LD^r(273\,nm)/LD^r(337\,nm)$, Eq. (6) allows a higher precision for comparing relative changes between the two order parameters. For absolute size Eq. (7) and (8) have been used, after scaling with S_D. In this way a physical interpretation of how the microscopic orientation parameters are affected by the interactions in the membrane 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 mole% of cholesterol. Pyrene shows negative LD peaks at 337 nm and 240 nm consistent with a preferred orientation of its longest in-plane symmetry axis (z) parallel to the lipid bilayer chains (Figure 1).

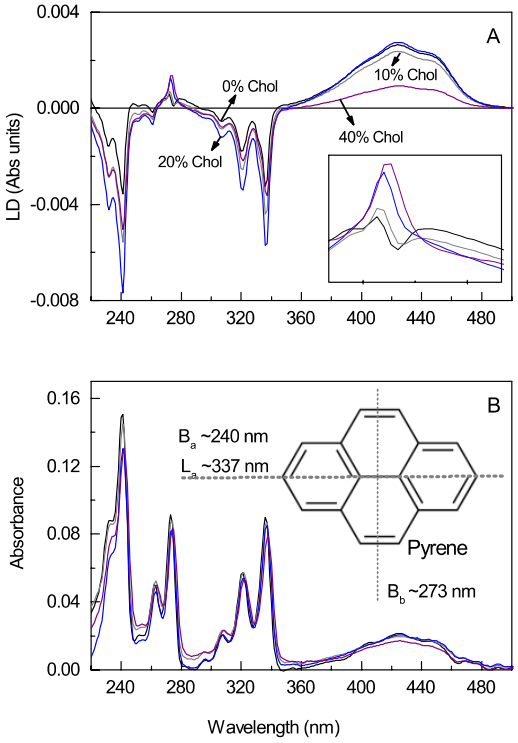

Figure 1. LD at a shear rate of 3100 s^{-1} (A) and absorbance (B) spectra of pyrene and curcumin in DOPC liposomes with different mole% of cholesterol: 0 (black line); 10 (gray); 20 (blue) and 40% (purple). Inset in A: LD band at 270-280 nm zoomed in. Inset in B: directions of the transition moments of pyrene.

Since pyrene is quite sensitive to its environment it was judged meaningful to carefully compare the absorption and LD spectra (Figure 1B). The LD spectrum of the L_a transition shows a characteristic sharp vibrational structure indicating that all pyrene molecules aligned are in a non-polar 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 smaller and positive in the more well-ordered system containing cholesterol, but appears both as positive and as negative peak, split in wavelength with the positive peak at slightly shorter and the negative at longer wavelength (inset in Figure 1A). 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 an "ambivalent" orientation, the plane of the molecule gives to the *y*-axis a certain prevalence for being aligned parallel with the lipid chains, while any orientation of the *z*-axis parallel with the lipid chains will, of course, make *y* perpendicularly oriented.[12] When comparing the reduced linear dichroism (LD^r) 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 2). For the sample with 40% cholesterol, it is the LD^r values at 273 nm versus those of curcumin at 424 nm that show a linear behavior (Figure 2).

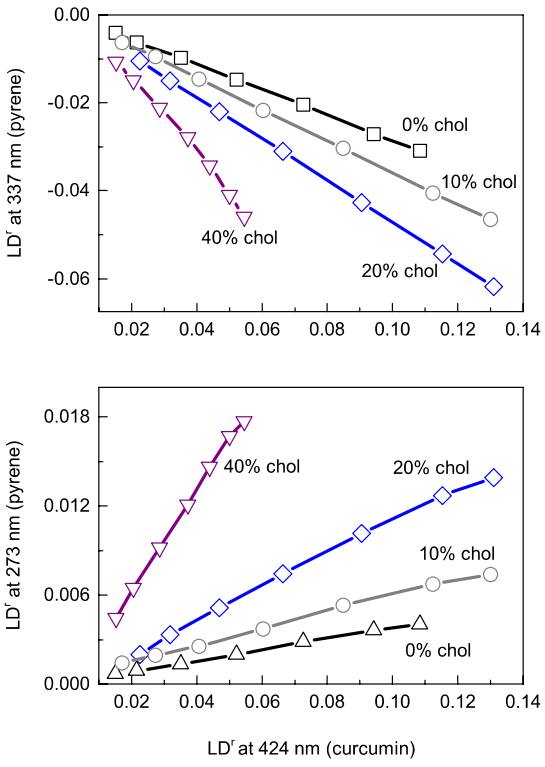

Figure 2. LD^r values of pyrene at 337 and 273 nm plotted versus the LD^r of curcumin at 424 nm in DOPC:cholesterol liposomes as a function of the shear rate (the symbols from left to right correspond to 80, 160, 320, 640, 1300, 2600, 3100 s^{-1}).

The linearity 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)-(8). The orientation parameter S_D is comparable for DOPC liposomes containing 0, 10 and 20 mole% of cholesterol, whereas the liposomes with 40% cholesterol are the poorest aligned showing the lowest S_D values in absolute numbers (Figure 3).

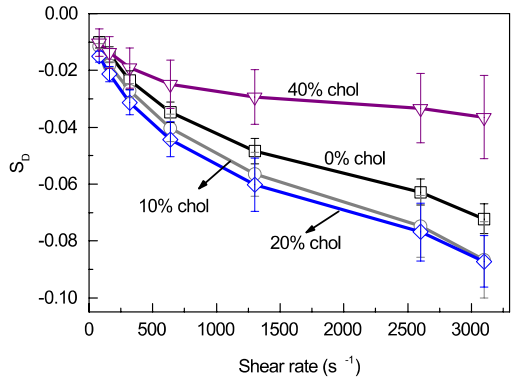

Figure 3. Orientation factor S_D of DOPC:cholesterol liposomes versus shear rate (symbols from left to right: 80, 160, 320, 640, 1300, 2600, 3100 s^{-1}).

The first strong transition (L_a) of pyrene, with a vibrational structure between 300-340 nm, has a pure polarization 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 a similar intensity as 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 1A, however, the corresponding LD is close to zero, indicating that S_{yy} is near zero (Figure 4). In the presence of cholesterol the tendency for alignment of the *z*-axis parallel to the lipid chains increases as seen from increasing of S_{zz} (Figure 4). 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 so to speak statistically balance each other: molecules with negative LD, which are red-shifted by a few nm, and others with positive LD which absorb a few nm to the blue compared to the center of the absorption maximum at 273 nm. The *S*-shaped LD is an effect of inhomogeneous broadening which is revealed thanks to that the infinitesimal spectral shifts are coupled to orientations. Note that 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 at 237 nm in combination with the red-shift is consistent with that the B_b (*y*-polarized) transition moment is more parallel with the lipid chains (which direction has the largest polarizability), whereas the positive LD is consistent with a perpendicular orientation 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 a near disk-like behavior favoring both orientations of the in-plane short axis as well as the long axis parallel with the membrane normal *D*.

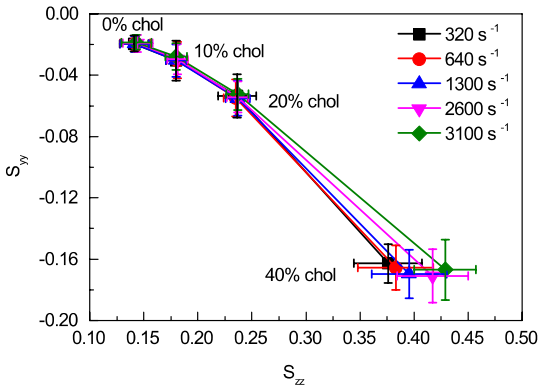

Figure 4. The microscopic order parameters S_{yy} and S_{zz} of pyrene in DOPC:cholesterol liposomes.

The split LD at 237 nm may tell something about the orientation distribution: the negative LD can be seen as corresponding to a sub-fraction of the molecular ensemble having positive S_{yy} and the positive LD to a fraction with negative S_{yy} values. Expressed 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 with the membrane normal (disk-like orientation) and the long axis to have this orientation (rod-like orientation). A similar near disk-like behavior with respect to the orientation direction of alkyl chains is also observed for pyrene when solubilized in a stretched polyethylene matrix.[12] This ambiguous disk/rod behavior is markedly changed by the presence of cholesterol. The S_{yy} decreases to more negative values while S_{zz} gets bigger. This is indeed the expected behavior for a better-oriented system since an increased S_{zz} will statistically make the *y*-axis more perpendicular and so S_{yy} more negative. Our results are in agreement with MD simulations of pyrene in a POPC membrane.[13] That study also shows that the *y*-axis of pyrene has a rather broad orientation distribution relative to the normal to the membrane plane whereas the *x*-axis shows a clear preference perpendicular to the membrane normal. With increasing cholesterol concentration, this distribution is predicted to sharpen up further. Such a behavior is expected if repulsive forces by steric crowding (rigid steroid skeleton) would further force the plane of the molecule to align parallel with the lipid chains and is consistent with cholesterol having an important role in ordering the lipid acyl chains. Cholesterol depletion in cells was actually found to disrupt the orientation of a fluorophore located within the hydrophobic region of the membrane but not of a surface bound probe.[9] Both dispersive and steric forces will favor an orientation of the longest dimension of pyrene parallel with the lipid chain direction, due to a maximum polarizability and a 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 with 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 pyrene long axis (S_{zz}) and short axis (S_{yy}).

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REFERENCES

(1) Janmey, P. A.; Kinnunen, P. K. *Trends Cell Biol* **2006**, *16*, 538.
(2) van Meer, G.; Voelker, D. R.; Feigenson, G. W. *Nat Rev Mol Cell Bio* **2008**, *9*, 112.
(3) Mitra, A. K.; Celia, H.; Ren, G.; Luz, J. G.; Wilson, I. A.; Teyton, L. *Current biology : CB* **2004**, *14*, 718.
(4) Edmiston, P. L.; Lee, J. E.; Wood, L. L.; Saavedra, S. S. *J Phys Chem* **1996**, *100*, 775.
(5) Tronin, A.; Strzalka, J.; Chen, X.; Dutton, P. L.; Blasie, J. K. *Langmuir* **2000**, *16*, 9878.
(6) Axelrod, D. *Biophysical journal* **1979**, *26*, 557.
(7) Ardhammar, M.; Mikati, N.; Norden, B. *J Am Chem Soc* **1998**, *120*, 9957.
(8) Brattwall, C. E. B.; Lincoln, P.; Norden, B. *J Am Chem Soc* **2003**, *125*, 14214.
(9) Benninger, R. K.; Onfelt, B.; Neil, M. A.; Davis, D. M.; French, P. M. *Biophysical journal* **2005**, *88*, 609.
(10) Langkilde, F. W.; Thulstrup, E. W.; Michl, J. *J Chem Phys* **1983**, *78*, 3372.

(11) Svensson, F. R.; Lincoln, P.; Norden, B.; Esbjorner, E. K. *Journal of Physical Chemistry B* **2007**, *111*, 10839.
(12) Thulstrup, E. W.; Michl, J.; Eggers, J. H. *J Phys Chem* **1970**, *74*, 3868.
(13) Loura, L. M. S.; do Canto, A. M. T. M.; Martins, J. *Bba-Biomembranes* **2013**, *1828*, 1094.