Chromophore Orientation in Liposome Membranes Probed with Flow Dichroism

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Liposomes can be considered models of cell membranes, and be used for studying transport and signal mechanisms of membrane proteins in situ, and are also used for drug delivery and as transfecting agents in gene therapy. Despite detailed structural information about isolated membrane proteins as obtained when assembled in crystal[1], there is still a lack of methods suitable for the study of proteins incorporated in cellular membranes. The present study demonstrates a simplistic approach to obtain information about preferred orientation of membrane constituents upon incorporation in liposome bilayers.

Absorption anisotropy (linear dichroism) has long been used to probe orientation in macroscopically aligned samples of molecules whose transition moment directions in a molecular frame are known. Examples include orientation of nucleobases and ligands in flow-oriented nucleic acids[2] as well as orientation of chlorophyll in assembled membranes.[3] Herein we show that the flow-orientation technique can be applied also to liposomes, due to the fact that the shear forces deform the normally spherical liposome into an ellipsoid with the major axis preferentially pointing at an angle less than 45° to the flow lines.[4] Using a phase-modulation technique,[5] the linear dichroism signal from aromatic molecules solubilized in the lipid bilayer of the liposome could be sensitively detected and interpreted in terms of a (macroscopic) orientation of the corresponding transition moments. For a transition polarized along the long axis of a molecule which has a preferred orientation parallel to the lipid hydrocarbon chains, a negative linear dichroism $LD = A_{\parallel} - A_{\perp}$ is expected; as shown by the sketch in Figure 1a, a prolate liposome will display a larger number of lipid chains oriented perpendicularly to its major axis than parallel to it. In qualitative agreement with this expectation, elongated aromatic molecules such as anthracene, pyrene, and perylene (Figure 1c, Table 1) are all found to exhibit negative LD signals at the wavelengths for electronic transitions polarized along their long axes (*z* axis), for pyrene shown in Figure 2. By contrast, in-plane short axis polarized transitions show positive LD, see Figure 1b and Figure 2. Perylene shows an interesting feature, both the long axis (*z*) and the short axis (*y*) transitions exhibit negative LD, thus indicating an orientation of both axes along the hydrocarbon chains, or more precisely, their bisector exhibits an orientation axis in the *xy* plane. The relative sizes of the reduced linear dichroism for the transitions (Table 1) suggest the orientation axis to be at an angle of approximately 20° to the *z* axis, a result in good agreement with the orientation of perylene observed in streched polyethylene films at room temperature.[6]

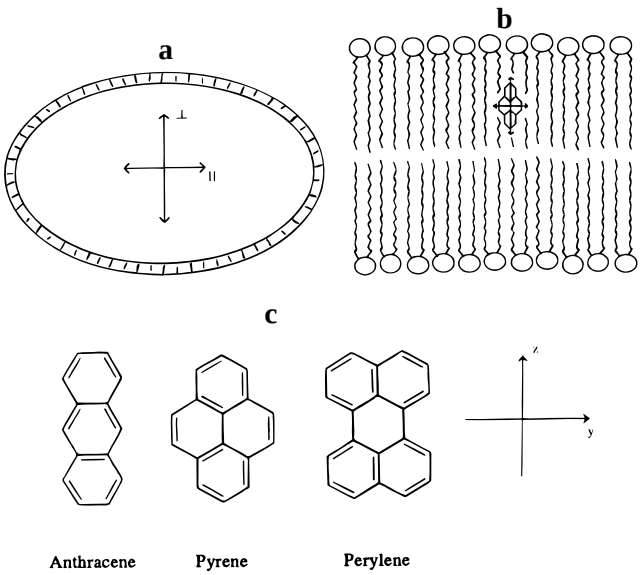

Figure 1. Liposome membrane with solubilized chromophores. (a) Liposome deformed by a linear shear flow. Directions of incoming polarized light indicated by arrows. Linear dichroism defined as differential absorption of linearly polarized light: $LD = A_{\parallel} - A_{\perp}$. (b) Section of a liposome membrane with a solubilized pyrene molecule. Arrows indicate short and long axis polarized transition moments. (c) The membrane chromophores anthracene, pyrene, and perylene with axis notations for short and long axis polarized transition moments.

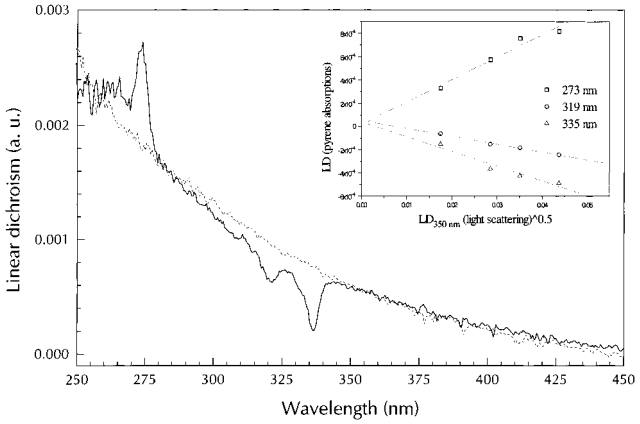

Figure 2. Flow LD spectrum of liposomes containing solubilized pyrene at a shear gradient of 3100 s^{-1}. Dashed curve shows LD of liposomes in the absence of pyrene. Liposomes were prepared by mixing soybean lipids and chromophores (molar ratio approximately 100:1) in the presence of hexane or chloroform. The solvent was then evaporated. To the lipid mixture was added 5 mM phosphate buffer (pH 7) to a final concentration of lipid less than 5 mg/mL. Liposomes were then prepared following a standard extrusion procedure.[9] Quasi-elastic light scattering showed liposome diameters of approximately 100 nm. Insert: LD from pyrene absorption grows approximately as the square root of the light-scattering anisotropy when increasing the shear rate.

The quantitative assessment of the orientation of the solute molecules relative to the membrane requires knowledge about the degree of alignment of the latter. Following standard analysis,[2] it can be shown that the linear dichroism normalized with respect to the absorbance (A_{iso}) of the same sample at isotropic conditions (no flow) is factorizable into one part ($S_{ma}S_N$) gauging the degree of orientation and deformation of the liposome

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Table 1. Reduced Linear Dichroism (LD^r) of Long Axis (*z*) and Short Axis (*y*) Polarized Transitions of Pyrene, Perylene, Anthracene, and Triphenylene Solubilized in Liposome Membranes at a Shear Gradient of 3100 s^{-1}

chromophore	$LD^r = LD/A_{iso} \times 10^3$ (wavelength in parenthesis) *z* polarization	*y* polarization	main chromophore orientation with respect to membrane normal *N*
pyrene	−1.6 (335 nm)	+2.9 (275 nm)	*z* parallel to *N*
perylene	−4.1 (254 nm)	−1.4 (435 nm)	*zy* bisector parallel to *N*
anthracene	−2.5 (252 nm)	+4.5 (374 nm)	*z* parallel to *N*
triphenylene	+0.8 (258 nm)		*zy* plane perpendicular to *N*

(see below) and one part (*Y*) depending on the orientation of the solute molecule relative to a local axis (*N*) perpendicular to the membrane surface

$$LD/A_{iso} = S_{ma}S_N Y \quad (1)$$

$S_{ma}S_N$ can be determined independently from measurement of light scattering of the flow-oriented liposomes (see below). The factor *Y* is given by the expression[5,6]

$$Y = 3(S_{zz}\epsilon_z + S_{yy}\epsilon_y + S_{xx}\epsilon_x)/(\epsilon_z + \epsilon_y + \epsilon_x) \quad (2)$$

where ϵ_z, ϵ_y, ϵ_x are, respectively, the extinction coefficients for light absorption along the molecule-fixed axes *z* (long axis), *y* (in-plane short axis), and *x* (out-of-plane axis). S_{zz}, S_{yy} and S_{xx} are corresponding orientation parameters defined as $S_{zz} = (^1/_2)(3\langle\cos^2\Theta_z\rangle - 1)$ with Θ_z the angle between the molecule axis *z* and the membrane normal (N), etc., and $\langle\ \rangle$ denotes the average over the angular distribution of solute molecules with respect to N. Two orientation parameters will be enough to specify the orientation of the molecule in the membrane since $S_{zz} + S_{yy} + S_{xx} = 0$.

S_{ma} of eq 1 may be obtained from LD/A_{iso} measured at wavelengths outside the absorption region of the chromophores, where the LD is due to turbidity. Following the principles of ref 7, the turbidity dichroism of an ellipsoidal shell, in absence of absorption, can be shown to approximate as

$$LD/A_{iso}\ (\text{turbidity}) = (\tau_{||} - \tau_{\perp})/\tau_{iso} \approx S_{ma}\{128\pi^2/75(r_2/\lambda)^2(1 - r_2/a_2)(1 - f^{5/3})\}/\{8/3(1 - f) - 64\pi^2/15(r_2/\lambda)^2(1 - f^{5/3})\} \quad (3)$$

where S_{ma} is the liposome orientation factor, $S_{ma} = 0.5[3\langle\sin^2\theta\rangle - \langle\sin^2\theta\cos 2\phi\rangle - 2]$, with θ and ϕ the angles defining the orientation of the liposome major axis relative to the direction of shear flow and of incidence and polarization of light.[7] $f = (r_1/r_2)^3$ is the cube of the ratio between the inner (r_1) and outer (r_2) radii defining the spherical liposome shell, λ is the wavelength of light and a_2 is the (outer) half major axis of the ellipsoidally deformed liposome. The derivation of eq 3 is included as Supporting Information to this communication.

S_N in eq 1 is the surface orientation function $(3\langle\cos^2\nu\rangle - 1)/2$, where ν is the angle between the major axis of the liposome and the normal N to the membrane, the average taken over the whole surface of the liposome. For an ellipsoidal liposome with an axial ratio *a*/*b* between major and minor axis, S_N may be approximated as

$$S_N = (b/a - 1)/2 \quad (4)$$

($S_N = 0$ for a sphere and $-1/2$ for an infinite cylinder).

The small positive turbidity dichroism $(\tau_{||} - \tau_{\perp})/\tau_{iso}$, typically in the range 0.0010−0.0030, indicates low values of both S_{ma} and S_N. For an axial relation $a/b \approx 1.4$, we obtained consistency with eq 3 with $S_{ma} \approx 0.06$, and $S_N \approx -0.15$ from eq 4 at a shear rate of 3100 s^{-1}. The value of *Y* in eq 1, and hence S_{zz} and S_{yy} in eq 2, are correspondingly larger, with pyrene LD/A_{iso} values equal to −0.0016 and +0.0029 at, respectively, 335 nm (pure *z* polarized) and 275 nm (pure *y* polarized) were recorded, thus corresponding to $S_{zz} = +0.06$ and $S_{yy} = -0.12$. The larger negative value of S_{yy} may seem unexpected for symmetry reasons, had pyrene been perfectly oriented along the lipid carbon chains ($\theta_z = 0°$, $\theta_y = \theta_x = 90°$), $S_{zz} = +1$, $S_{yy} = S_{xx} = -0.5$. On the other hand, if the pyrene molecule is positioned flat at the surface ($\theta_z = \theta_y = 90°$ and $\theta_x = 0°$), $S_{zz} = S_{yy} = -0.5$ and $S_{xx} = +1$. Thus, the observation of a large negative S_{yy} can only be explained if a certain fraction of the pyrene molecules is aligned parallel to the surface of the membrane and the major fraction is aligned parallel to the lipid hydrocarbon chains. From the knowledge of typical pyrene orientation in ordered alkane chain matrix[5] (polyethylene: $S_{zz} = +0.25$, $S_{yy} = -0.01$), we estimate the latter fraction to be about 75%. The "surface mode" of adsorption was also observed with a disklike molecule, triphenylene, and is consistent with the observed location of benzene at the surface of micelles.[8]

Our results indicate that the degree of orientation and deformation of lipid vesicles is rather small even at high shear fields, in agreement with hydrodynamic models,[4] whereas the microscopic orientation of solute chromophores in the membrane is very efficient. As seen from the insert of Figure 2, the linear dichroism from pyrene absorption grows approximately as the square root of the light scattering anisotropy when increasing the shear rate, reflecting the different functional dependences of absorption anisotropy (eq 1) and turbidity anisotropy (eq 3).

Supporting Information Available: Derivation of eq 3 (2 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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