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Does Aliphatic Chain Length Influence Carbocyanines' Orientation in Supported Lipid Multilayers?

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UV-Vis linear dichroism was used to study the orientation of 3,3′-dihexyloxacarbocyanine ($DiOC_6(3)$) and 3,3′-dihexadecyloxacarbocyanine ($DiOC_{16}(3)$) in supported multilayers of dipalmitoylphosphatidylcholine (DPPC) and dilauroylphosphatidylcholine (DLPC). Orientational probability density functions were similar for the two carbocyanines in both lipids. Multimodal distributions were found in all cases. The main peak is at 9°–11° relative to the bilayer normal axis, except for $DiOC_{16}(3)$ in DLPC multilayers (main peaks at 13° and 90°). Quenching studies revealed that the two carbocyanines are localized at the interface region of the membrane regardless of the lipid matrix they are inserted in. Combining these data with linear dichroism results lead to the conclusion that both the aliphatic chain length of carbocyanines and the lipid phase have little influence in the structural organization of these probes in lipidic bilayers. The partition constants of $DiOC_6$ (3), K_p, were determined from fluorescence anisotropy measurements; the values obtained were K_p (DPPC) $= (2.39 \pm 0.05) \times 10^3$ and K_p (DLPC) $= (5.01 \pm 1.15) \times 10^3$.

KEY WORDS: Carbocyanines; UV-Vis linear dichroism; orientation.

INTRODUCTION

Cyanine dyes have been widely used to study biological systems. They are commonly used to investigate different lipid environments [1] and in studies of macromolecules' aggregates such as DNA [2]. The large variety of existing dyes and the low cost of some made them commonly used probes. The most popular carbocyanine membrane probes have been indocarbocyanines (DiI) and oxacarbocyanines (DiO) with long alkyl tails (C_{18}). Having strong oscillator strengths for electronic absorption, being weakly fluorescent in water but highly fluorescent and photostable when inserted in lipidic membranes, makes them good and appealing to work in model systems of biomembranes by means of fluorescence spectroscopy.

Although these probes are widely used to report properties of the lipidic systems where they are inserted in, little attention has been given, to our best knowledge, to the oxacarbocyanines dyes themselves, mainly when short alkyl chains are used. It is intuitively expected that the results generally obtained with cyanines from such studies may depend on the probe's location and orientation but systematic studies are very scarce [3]. Nevertheless, ⟨⟨Identification of solubilization sites for the dye in the membrane and its local structure (site of solubilization, surface vs. core, and orientation with respect to the interface) is a prerequisite for an understanding of the molecular mechanism for the change in the fluorescence parameters⟩⟩, as pointed out before by Krishna and Periasamy [4].

In this paper we focused our attention in two oxacarbocyanines, $DiOC_{16}(3)$ and $DiOC_6(3)$ (Fig. 1A and B), which differ only in their aliphatic chain length. A comparative location and orientation study was carried out by means of fluorescence quenching and UV-Vis linear dichroism techniques. Using different lipids, we

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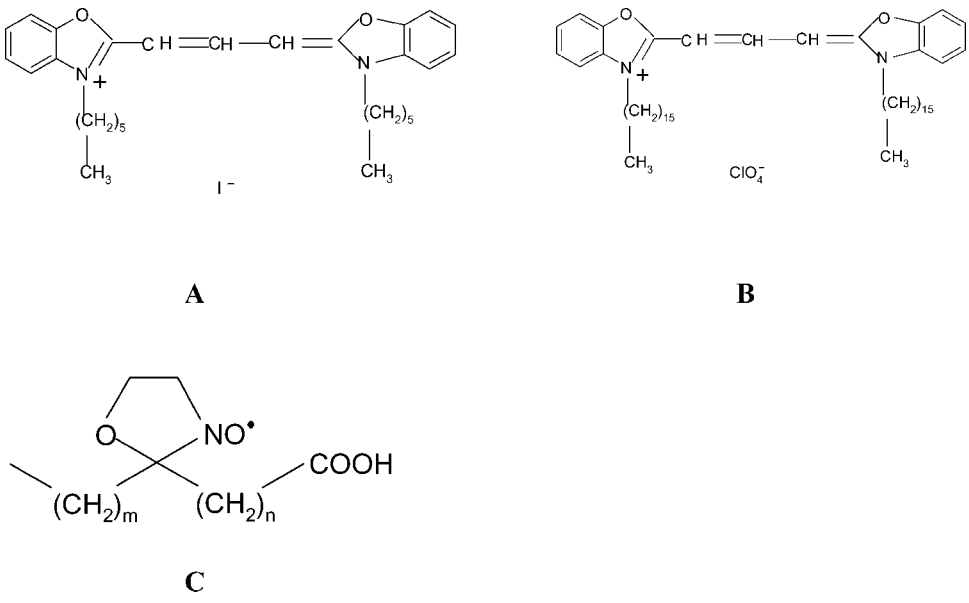

Fig. 1. Structures of 3,3′-dihexyloxacarbocyanine ($DiOC_6(3)$) iodide (A), 3,3′-dihexadecyloxacarbocyanine ($DiOC_{16}(3)$) perchlorate (B), and doxyl-stearic acids (5NS: $n = 3$, $m = 12$; 16NS: $n = 14$, $m = 1$) (C).

concluded on how chain length and the lipid phase influence the location and orientation of these probes.

EXPERIMENTAL

3,3′-Dihexyloxacarbocyanine ($DiOC_6(3)$) iodide, 98%, was from Acros Organics (Gell, Belgium) and 3,3′-dihexadecyloxacarbocyanine ($DiOC_{16}(3)$) perchlorate, 99%, was from Molecular Probes (Oregon, USA). DPPC and DLPC were from Avanti Polar Lipids (Alabaster, AL) and both 5-doxyl-stearic acid (5NS) and 16-doxyl-stearic acid (16NS) were from Aldrich Chem (Milwaukee, USA). All solvents were from Merck (Darmstadt, Germany) with spectroscopic grade.

UV-VIS LINEAR DICHROISM (ORIENTATION) STUDIES

Samples of aligned lipids multilayers were obtained by incomplete evaporation of the aqueous solvent. First, multilamellar vesicles were prepared. The lipid solution in chloroform was dried in a round flask under a N_2 flow and placed in vacuum overnight. Then the lipid was ressuspended in Millipore water to a final concentration of 1.36 mM, by cycles of vortexing/heating. A drop of the suspension (25 μL) with or without carbocyanine (68 μM) was spread on one surface of a quartz plate and the solvent was slowly evaporated under a controlled N_2 flow with a pipette in order to make the film as uniform as possible. The final molar ratios of lipid to cyanine were 20:1. The structural organization of multilayers obtained by solvent evaporation has been checked before [5].

UV-Vis absorption measurements were carried out in a Shimadzu spectrophotometer (model UV-3101 PC). Dichroic ratios were determined using Glan-Thompson polarizers in home-made stands. Blank correction was carried out by reading both sample (lipid + carbocyanines) and blank (lipid) against air, and subtracting afterward. However, in DPPC/$DiOC_6(3)$ samples a more sophisticated additional process was needed due to turbidity, as described elsewhere [6]. This method is based on data extrapolation from wavelength ranges where only turbidity is operative. The usual sample holder was replaced by a goniometer (Optosigma, SL) where the quartz plates were mounted in. A spectrofluorimeter SLM-Aminco 8100 was also used, equipped with a 450 W Xe Lamp and double monochromators in both excitation and emission, as well as a quantum counter. Dichroic ratios and fluorescence intensity ratios were measured in the range of 0–72° and 18°–72° respectively. Excitation and emission wavelengths were, respectively, 494 nm and 547 nm.

A more detailed description of the data analysis methodologies can be found elsewhere [7]. Shortly, orientational distribution functions, $f(\psi)$ (Eq. (1)), are calculate from a Legendre polynomial series:

$$f(\psi) = \sum_{L_{even}} \frac{1}{2}(2L+1)\langle P_L \rangle P_L(\cos\psi) \qquad (1)$$

so that

$$\int_0^{\pi} \sin(\psi)\, f(\psi)\, d\psi = 1 \tag{2}$$

while

$$\langle P_L \rangle = \int_{-1}^{1} P_L(\cos\psi)\, f(\psi)\, d(\cos\psi) \tag{3}$$

($P_L(\cos\psi)$ are Legendre polynomials; $\langle P_L \rangle$ is the ensemble-average of $P_L(\cos\psi)$ and is referred to as the Lth rank order parameter, when $L \neq 0$).

As $f(\psi)$ is symmetrical ($f(\psi) = f(-\psi)$ and $f(\psi) = f(\pi - \psi)$), $\langle P_L \rangle = 0$ if L is odd. Therefore:

$$f(\psi) = \frac{1}{2} + \frac{5}{2}\langle P_2 \rangle P_2(\cos\psi) + \frac{9}{2}\langle P_4 \rangle P_4(\cos\psi) + \cdots$$

$$\left(P_2(\cos\psi) = \frac{3\cos^2\psi - 1}{2} \quad \text{and} \right.$$

$$\left. P_4(\cos\psi) = \frac{35\,\cos^4\psi - 30\,\cos^2\psi + 3}{8} \right) \tag{4}$$

because $P_0(\cos\psi) = 1$. For an isotropic system, $\langle P_L \rangle = 0$ if $L \geq 2$ and $f(\psi) = 1/2$. For perfectly aligned molecules, $\langle P_L \rangle = 1$ if $L \geq 2$ and $f(\psi) = \delta(\cos\psi - 1)$ (δ is Dirac's delta function). Intermediate distributions are characterized by their peculiar set of $\langle P_2 \rangle$, $\langle P_4 \rangle$, $\langle P_6 \rangle \ldots$ However, only $\langle P_2 \rangle$ and $\langle P_4 \rangle$ can be known from experiment. Thus, the quest for $f(\psi)\sin(\psi)$ (Probability Density Function) resumes to two steps: (1) $\langle P_2 \rangle$ and $\langle P_4 \rangle$ determination, and (2) finding an approximated function for $f(\psi)\sin(\psi)$ from $\langle P_2 \rangle$ and $\langle P_4 \rangle$ only. The most common realistic approximation combines the application of the maximum entropy method with the Lagrange multipliers method [8]. In this approach, the approximated function is the one that maximizes the orientational entropy of the distribution, $S(f(\psi))$:

$$S(f(\psi)) = \int_0^{\pi} \ln(\psi)\sin(\psi)\, f(\psi)\, d\psi \tag{5}$$

In other words, the resulting distribution is the broadest possible from all the universe of distributions having that particular ($\langle P_2 \rangle$, $\langle P_4 \rangle$) pair:

$$f(\psi) = A \exp\left(\sum_L \lambda_L P_L(\cos\psi) \right) \tag{6}$$

A is a normalization constant and the λ_L coefficients (Lagrange multipliers) are calculated from the accessible $\langle P_L \rangle$ values:

$$\langle P_2 \rangle = \int_0^{\pi} \sin\psi\, P_2(\cos\psi)\, f(\psi)\, d\psi \tag{7}$$

$$\langle P_4 \rangle = \int_0^{\pi} \sin\psi\, P_4(\cos\psi)\, f(\psi)\, d\psi \tag{8}$$

$$f(\psi) = \frac{\exp(\lambda_2 P_2(\cos\psi) + \lambda_4 P_4(\cos\psi))}{\int_0^{\pi} \sin\psi \exp(\lambda_2 P_2(\cos\psi) + \lambda_4 P_4(\cos\psi))\, d\psi} \tag{9}$$

$$f(\psi)\sin\psi = \frac{\exp(\lambda_2 P_2(\cos\psi) + \lambda_4 P_4(\cos\psi))}{\int_0^{\pi} \sin\psi \exp(\lambda_2 P_2(\cos\psi) + \lambda_4 P_4(\cos\psi))\, d\psi} \sin\psi \tag{10}$$

The probability density function, $f(\psi)\sin(\psi)$, is then easily obtained.

QUENCHING (IN-DEPTH LOCATION) STUDIES

Quenching studies were carried out using DPPC and DLPC Large Unilamellar Vesicles (LUV's), prepared by extrusion techniques as described in [9], with cyanine incorporated to a final lipid:cyanine 830:1 ratio. 5NS or 16NS (in ethanol) (Fig. 1C) were added to these suspensions to a final concentration of 0.42 M inside the lipidic matrix. The cyanine's fluorescence intensity was read ten minutes after each addition. The final percentage of ethanol in the solution was kept below 2% in order to guarantee that there was no damage in the prepared vesicles. A more detail description for the interpretation of quenching results can be found in reference [10]. Briefly, it is based in the dependence of quenching extent on the local concentration of the quencher in the vicinity of the fluorophore. When acyl chains derivatized at shallow position are better quenchers than the ones derivatized near the bilayer core terminus (e.g. 5NS relative to16NS), a location of the fluorophore near the interface can be inferred. The opposite result leads to the opposite conclusion.

PARTITION STUDIES

Partition studies with $DiOC_6(3)$ were carried out using different DPPC and DLPC Large Unilamellar Vesicles (LUV's) concentration (until a final concentration of 5 mM was reached) with a fixed cyanine concentration of 7 μM. Steady-state fluorescence anisotropy was measured at $\lambda_{exc} = 494$ nm and $\lambda_{em} = 514$ nm. The measured steady-state fluorescence anisotropy, $\langle r \rangle$, is a balance between the anisotropy of cyanines in bulk aqueous phase and cyanines inserted in the lipidic matrix. The weight factors in this balance depend on the partition coefficient, K_p, wich was calculated as a fit parameter in a non-linear regression methodology. Details regarding this methodology can be found elsewhere [11].

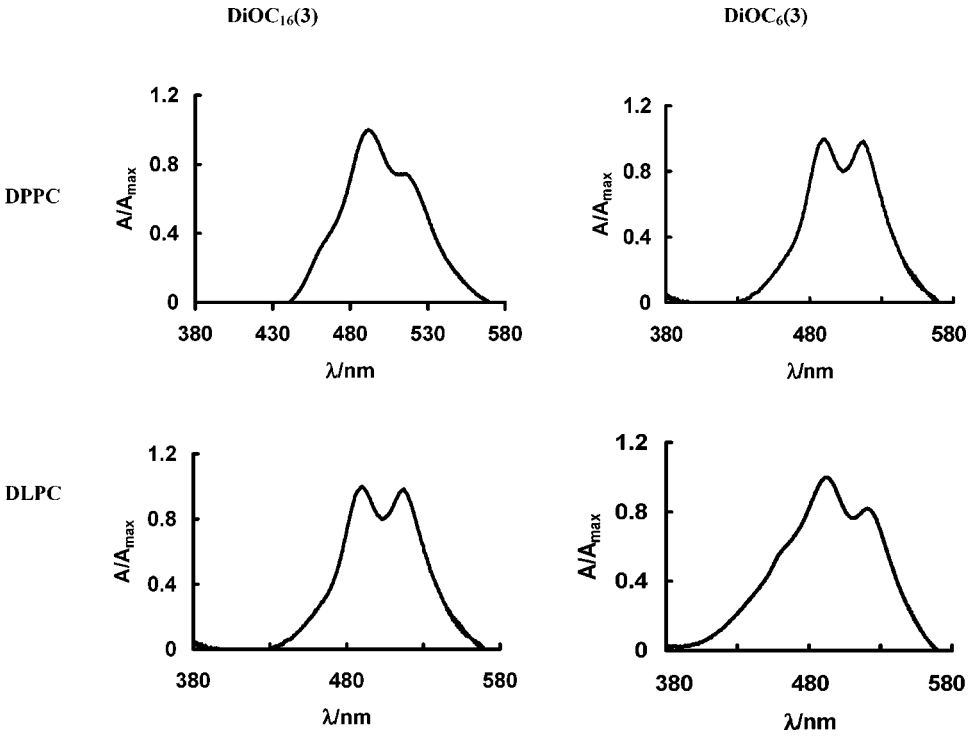

Fig. 2. $DiOC_6(3)$ and $DiOC_{16}(3)$ normalized absorption spectra in supported lipid multilayers of DPPC and DLPC (ratio lipid:cyanine 20:1) at room temperature.

For $DiOC_{16}(3)$ complete incorporation in the lipid matrix was assumed, which is in agreement with its very low solubility in aqueous media, since similar probes present very big partition coefficients [1].

RESULTS AND DISCUSSION

The absorption spectra of both cyanines ($DiOC_6(3)$ and $DiOC_{16}(3)$) in DPPC and DLPC multilayers are depicted in Fig. 2. The vibrational progression of the absorption spectra in the aligned multilayers is similar to the one obtained when cyanines are inserted in vesicles. Both cyanines have a transition moment that is fairly parallel to the chromophore axis, which enables direct molecular orientation study of the chromophore. The second and fourth rank order parameters, $\langle P_2 \rangle$ and $\langle P_4 \rangle$ respectively, and Lagrange multipliers (λ_2 and λ_4) needed for the calculation of orientational distribution function are presented in Table I. The probability density functions calculated with these parameters are shown in Fig. 3. Optimisation of the signal/noise ratio was obtained with 20:1 molar lipid:cyanine ratio, which is high compared to methodologies based exclusively in fluorescence spectroscopy techniques [3,4]. Multimodal functions were obtained for the different systems but this cannot be regarded as a consequence of the presence of cyanine dyes in the water layers between the lipidic bilayers. The partition coefficients, even for $DiOC_6(3)$ (K_p (DPPC) $= (2.39 \pm 0.05) \times 10^3$ and Kp (DLPC) $= (5.01 \pm 1.15) \times 10^3$; Fig. 4) are favourable to the lipidic phase. As the aqueous and lipidic layers have similar volumes in supported multilayers, the molar fraction of dyes inserted in lipids, χ_L, are bigger than 99%. Quenching studies revealed that the two oxacarbocyanines are localised at the interface region of the membrane regardless of the matrix lipid they are inserted in (results not shown).

Table I. Second and Fourth Rank Order Parameters for Each of the Studied Systems[a]

System	$\langle P_2 \rangle$	$\langle P_4 \rangle$	λ_2	λ_4
DPPC:$DiOC_6(3)$ (20:1)	0.460	0.450	0.786	2.624
DLPC:$DiOC_6(3)$ (20:1)	0.571	0.569	0.730	3.508
DPPC:$DiOC_{16}(3)$ (20:1)	0.580	0.512	1.116	2.717
DLPC:$DiOC_{16}(3)$ (20:1)	0.280	0.317	0.524	2.027

[a] λ_2 and λ_4 are Lagrange multipliers that result from the application of the maximum entropy method to obtain the orientational distribution functions (see reference [7])

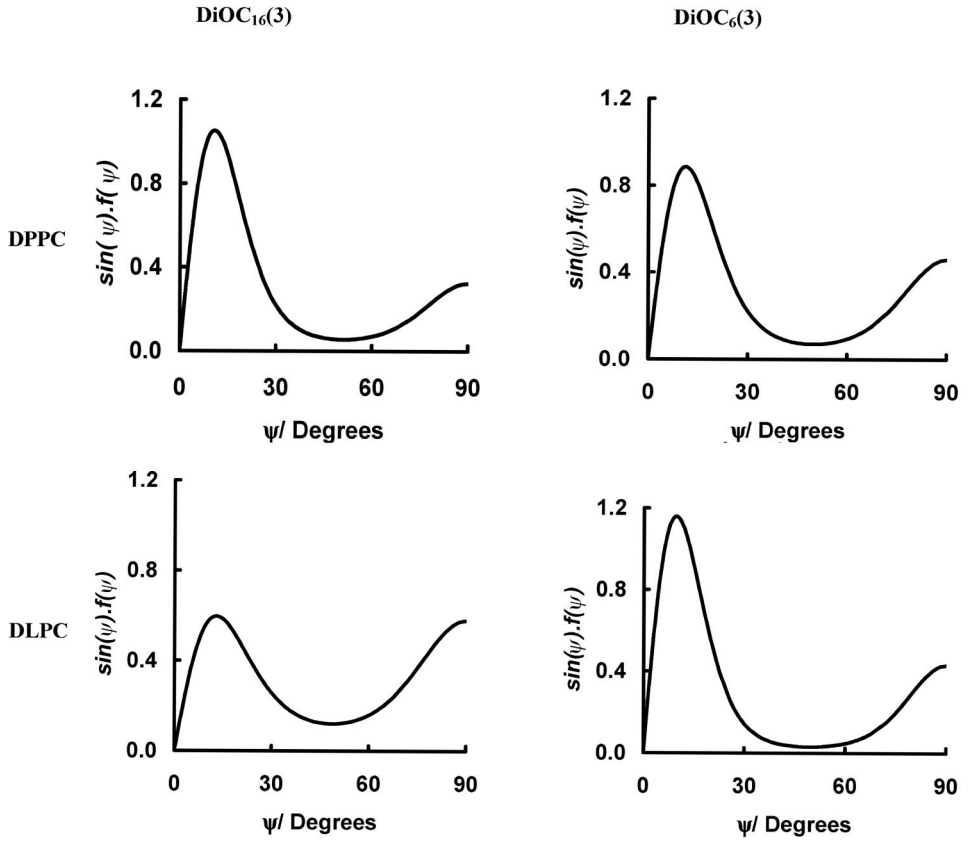

Fig. 3. Orientational probability density functions for $DiOC_{16}(3)$ and $DiOC_6(3)$ in DPPC and DLPC multilayers at room temperature.

In all the cases, but especially for the system DLPC/$DiOC_{16}(3)$, the peaks at 90° are significant and deserve some attention. One can point several reasons to explain the results: (1) a fraction of the chromophores lies parallel to the bilayers plane, (2) a fraction of the bilayers has macroscopic defects and involves cyanine molecules, (3) the peaks are spurious and result from the data analysis method. Previous studies with other probes [5,6] lead to the conclusion that multilayers are quite ordered and homogenous, which hardly accounts for such significant peak areas. The second hypothesis is thus considered not important. Reason number 3 is not likely at the used signal/noise ratios but cannot be excluded because orientational density probability functions are very sensitive to error in Lagrange multipliers λ_2 and λ_4; propagation into the 90° peak is mainly imposed by the sinus term in Eq. (10). Reason number 1 is the most likeky. The DLPC/$DiOC_{16}(3)$ (i.e. long tail cyanine in short-tail fluid lipids) system is peculiar, with an almost equal balance between both peaks. This result is probably related to simultaneous occurrence of lipid/probe acyl chains mismatch and high orientational freedom conferred by the liquid crystalline DLPC matrix. In binary lipid mixtures, where the shorter chain phospholipids form fluid domains [12], interdigitation in the opposing leaflet of the bilayer of molecules having long alkyl chains leads such molecules to prefer gel-like domains [13]. Therefore, hypothesis number 1 cannot be ruled out. A bimodal orientational distribution of probes is possible, although orientations in the 5°–30° (relative to the bilayer normal axis) range are more likely to occur than 70°–90° in most systems. Using a methodology that balances two assumed sub-populations (parallel and perpendicular to the membrane surface) with a similar dye, Krishna and Periasamy [3] obtained bimodal functions with pronounced peaks at 90° from the bilayer normal.

Theoretical studies have not reached a definitive conclusion regarding the major force governing the in-depth distribution of molecular probes in lipid bilayers [5]. Nevertheless, we can say that weak van der Waals interactions are responsible for acyl chain packing and that the number of interacting molecules, present in lipid layers, is large enough to overcome the disrupting influence of

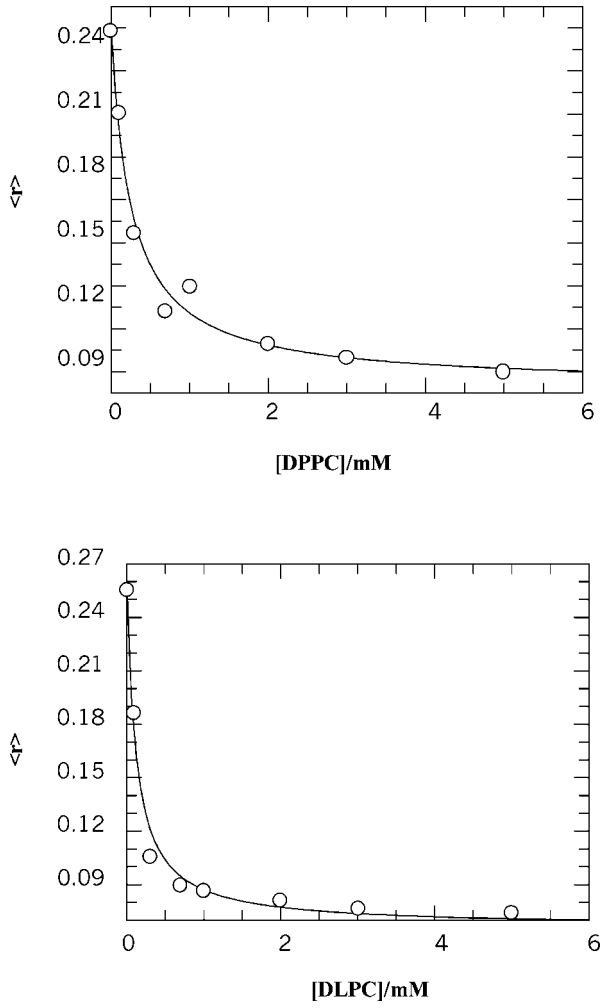

Fig. 4. Steady-state fluorescence anisotropy dependence on lipidic concentration for $DiOC_6(3)$ in DPPC and DLPC Large Unilamellar Vesicles at room temperature. Solid lines represent best fit to the data. Partition coefficients are obtained as fit parameters [11]. Anisotropy decreases due to an increase in fluorescence lifetime as partition occurs.

thermal agitation. Polar interactions predominate when accounting for the enhanced interfacial packing. When using forcefields to describe interactions of simulated biological macromolecules, the magnitude of electrostatic interactions is of the order of 60 kJ/mol, and that of the van der Waals interactions can assume values between 0–40 kJ/mol [14]. Considering the relative weight of these values for the case of small molecules like the carbocyanines, it is safe to expect that their positively charged chromophore group will interact strongly with the charged groups of the polar heads of DPPC and DLPC. These interactions will predominate over weak van der Waals interactions that the acyl chains may establish. This way, both carbocyanines present similar distributions with the favorable electrostatic interaction leading the chromophore group towards the vicinity of the lipid layer interface.

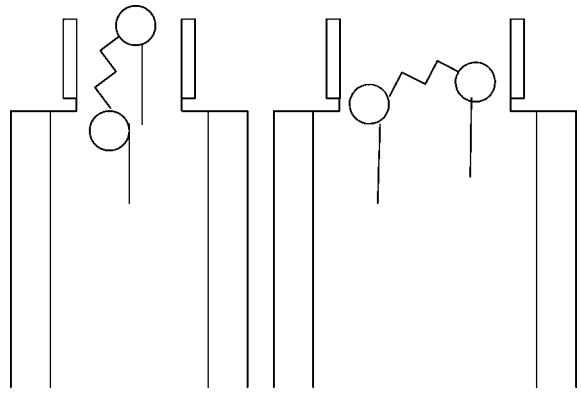

Fig. 5. Schematic representation of a oxacarbocyanine's orientation and localization between the polar headgroups of phospholipid molecules. Bimodal distributions were measured with peaks at 9°–11° in most experimental conditions. Alkyl chains are not drawn to scale and are merely illustrative. ◯ — Cyanines' ring groups; [] — Phospholipid headgroup; | — Aliphatic chain.

CONCLUSIONS

Our results revealed that both oxacarbocyanines insert extensively into lipidic membranes, where they are localized at the interface region. Their chromophores are mainly parallel to the chains of the lipids regardless its own chain length or the matrix lipid they are inserted in (Fig. 5). Probe/host chain mismatch can however lead to more complex distributions.

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