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Molecular dynamics simulations of DiI-C₁₈(3) in a DPPC lipid bilayer

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Abstract

We performed a 40 ns simulation of 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-C₁₈(3)) in a 1,2-dipalmitoyl-sn-glycero-3-phosphatidyl choline (DPPC) bilayer in order to facilitate interpretation of lipid dynamics and membrane structure from fluorescence lifetime, anisotropy, and fluorescence correlations spectroscopy (FCS). Incorporation of DiI of 1.6 to 3.2 mol% induced negligible changes in area per lipid but detectable increases in bilayer thickness, each of which are indicators of membrane structural perturbation. The DiI chromophore angle was $77 \pm 17^\circ$ with respect to the bilayer normal, consistent with rotational diffusion inferred from polarization studies. The DiI headgroup was located 0.63 nm below the lipid head group–water interface, a novel result in contrast to some popular cartoon representations of DiI but consistent with DiI's increase in quantum yield when incorporated into lipid bilayers. Importantly, the fast component of rotational anisotropy matched published experimental results demonstrating that sufficient free volume exists at the sub-interfacial region to support fast rotations. Simulations with non-charged DiI head groups exhibited DiI flip-flop, demonstrating that the positively-charged chromophore stabilizes the orientation and location of DiI in a single monolayer. DiI induced detectable changes in interfacial properties of water ordering, electrostatic potential, and changes in P–N vector orientation of DPPC lipids. The diffusion coefficient of DiI ($9.7 \pm 0.02 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$) was similar to the diffusion of DPPC molecules ($10.7 \pm 0.04 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$), supporting the conclusion that DiI dynamics reflect lipid dynamics. These results provide the first atomistic level insight into DiI dynamics, results essential in elucidating lipid dynamics through single molecule fluorescence studies.

Introduction

Structural organization of native lipids into bilayers is the result of a complex interplay of polar lipid head groups' interaction with each other, interfacial water, and the hydrophobic interactions of the lipid acyl chains.¹ Native lipids act collectively as a structured solvent of transmembrane and peripheral membrane proteins and modulate protein function through hydrophobic interactions,² lipidation,³ and bulk modulation of membrane viscosity.⁴ Interactions between lipids and proteins result in regulation of important cellular functions such as transmembrane ion transport,⁵ organization and modulation of signaling complexes,^{6,7} vesicle trafficking,⁸ energy conversion,⁹ and mechanosensation.^{10–12} Since

membrane lipid dynamics regulate membrane organization and lipid and protein-mediated signal transduction, studies on lipid dynamics and organization are at the center of understanding membrane function.

Popular tools to study lipid dynamics are fluorescence correlation spectroscopy (FCS), fluorescence recovery after photobleaching (FRAP), fluorescence anisotropy, and fluorescence lifetime. The spatial precision of these techniques is limited only by the diffraction limit of modern microscope objectives and wavelength and can thus reveal distributions of dynamics of single molecules in compartments in live cells.^{12–15} Each of these methods relies on mathematical models relating diffusion, rotation, or association to fluorescence arising from a probe that is either free (*e.g.*, diphenylhexatriene (DPH), carbocyanine dyes, or pyrene) or conjugated to various parts of lipid molecules (*e.g.*, pyrene phosphocholine, 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-phosphatidylcholine). However, since these fluorescence techniques only indirectly probe dynamics of the native lipids, there exists a need to precisely determine, at the atomic level, the dynamics and interactions of fluorescent probes with native lipids and water in order to assess fidelity of the probe to lipid dynamics, the extent to which the probe perturbs the bilayer, and to facilitate new insight into bilayer structure from dye fluorescence.¹⁶

One of the most popular dyes for membrane studies is dialkyl carbocyanine (DiI)¹⁷ because of its structural similarity to phospholipids and its ability to selectively partition into various phases of the membrane (gel phase *versus* liquid phase) depending on the length of the alkyl chains (C_N 10–22).¹⁸ DiIs are amphiphilic with a positively-charged head group chromophore consisting of two indole rings connected by an unconjugated double-bond carbon bridge. Two variable-length (C_N , $N \sim 10$ –22) saturated alkyl chains make up the non-polar tails. The absorption and fluorescence emission properties of DiIs can be adjusted by varying the hetero atoms of the indole rings or the length of the conjugated double bond carbon bridge.¹⁹ DiIs have high extinction coefficients, good fluorescence quantum yields, and short excited-state lifetimes in membranes.¹⁸ They have very low critical micelle concentrations in the aqueous phase and are only weakly fluorescent in water, but highly fluorescent and photostable when incorporated into membranes.¹⁹

Fluorescence spectroscopic analysis of DiI has been used to infer membrane lateral and rotational lipid mobility,²⁰ membrane potential,¹⁹ membrane fusion,²¹ fluorescence resonance energy transfer,²² phase separation,²³ lipid leaflet transmigration,²⁴ and the existence of lipid rafts.¹⁶ However, remaining uncertainty in precise location, orientation, hydration, and the surrounding environment of DiI,²⁵ have made interpretation of fluorescence lifetime, anisotropy, lateral and rotational dynamics difficult. For example, there is little agreement on the axial location of DiI relative to lipid head groups. Axial location is related to a number of photophysical properties of DiI including fluorescence lifetime and anisotropy decay. Use of fluorescence anisotropy revealed a fast rotational component of another lipid dye leading to the conclusion that the dye chromophore can exist near the surface of the bilayer well above the polar head groups of the surrounding native lipid molecules.^{26,27} In that study, it was assumed that steric hindrance of the base of the acyl chains of lipids would inhibit dye rotational diffusion. However, because water accessibility to the chromophore is expected to lead to quenching of fluorescence, this result is at odds with the high increase in quantum yield when DiI is incorporated into bilayers. Thus, remaining questions regarding the precise axial location and orientation of DiI in lipid bilayers can only be answered by methods which include all relevant inter- and intra-molecular forces with atomic spatial and picosecond temporal resolution.

Lipid systems can be studied using computational molecular dynamics (MD). MD uses Newtonian dynamics to follow the trajectories of all atoms due to inter- and intramolecular

interactions. These simulations are rich sources of quantitative data on molecular flexibility, lipid rotational and lateral diffusion, water dynamics and ordering, and atom–atom interactions. Although MD cannot simulate fluorescence directly because fluorescence is inherently a quantum mechanical process, MD analysis of fluorescent dyes can provide the atomic level mechanics and dynamics necessary to interpret membrane structure from fluorescence. For example, MD has been used to determine the location and orientation of DPH, pyrene, and NBD-PC in diphosphatidyl phosphocholine (DPPC).^{28–30} Interpretation of lipid structure and dynamics from fluorescence lifetime and anisotropy data is not straight-forward because the relative location and dynamics of the dye molecule is unknown. MD simulations can help resolve issues regarding location and dynamics of probe molecules within the bilayer which may lead to more accurate depiction of bilayer structure inferred from single molecule fluorescence.²⁸

In this study we performed MD simulations of DiI-C₁₈(3), (1,1'-dialkyl3,3,3',3'-tetramethylindocarbocyanine) in the fluid phase of a 1,2-dipalmitoyl-sn-glycero-3-phosphatidyl choline (DPPC) bilayer. First, we performed simulations with varying concentrations of DiI in order to examine the perturbative effects of DiI on the structural properties of the lipid bilayer. Second, we sought to clarify issues relating to the chromophore's axial location and distribution of orientations relative to lipids. Third, we detail the effects of DiI on interfacial water ordering, electrostatics, and P–N vector orientation, which are important parameters when interpreting fluorescence lifetime and quantum yield. Finally, the similarity of the lateral and rotational dynamics of DiI to lipid molecules was examined.

Methods

Three bilayers each with 128 molecules were built according to the method of Mukhopadhyay *et al.*³¹ with DiI :DPPC ratios of (i) 0 : 128 (“0-dye”), (ii) 2 : 126 (“2-dye”), and (iii), 4 : 124 (“4-dye”). A total of 4488 water molecules were added to each system. The hydration levels correspond to ~35 molecules of water to one molecule in the bilayer. A fourth simulation, similar to the 4-dye case, but in the absence of any partial charges on the DiI head group was also performed (“no charge”). In the 2-dye simulation, one DiI molecule was placed in each leaflet of the bilayer. In the 4-dye case, 2 DiI molecules were placed in each leaflet of the bilayer. The choice of the number of DiI molecules in the lipid bilayer was guided by the need to ensure symmetry of any perturbative effects of DiI on the lipid bilayer. The DiI concentration corresponded to 1.6 mol% (2 : 126) and 3.2 mol% (4 : 124) for the 2- and 4-dye cases, respectively. The concentrations of DiI were at the high limit compared to experiment (1 : 500 in the case of FRAP and 1 : 5000 in the case of FCS). Prior to all simulations, DiI molecule headgroups were placed *a priori* at a z-position of 2.3 nm from the center of the bilayer, which was 0.5 nm above the lipid headgroup–water interface (which was at a z-position of 1.8 nm). Upon 10 ns pre-equilibration, DiI headgroups migrated to equilibrium positions below the interface (to a z-position of 1.2 nm).

The united atom description was adopted for CH, CH₂ and CH₃ groups in the DPPC and DiI-C₁₈(3). The DPPC parameters for the bonded and non-bonded interactions were obtained from a study of a pure DPPC bilayer performed earlier by Berger *et al.*³² The data for that simulation are available at <http://moose.bio.ucalgary.ca/files/lipid.itp> and <http://moose.bio.ucalgary.ca/files/dppc.itp>. Partial charge description on DPPC were identical to those described by Chiu *et al.*³³ The initial structure for DiI-C₁₈(3) was constructed using the molecular building software, GAUSSVIEW, which is a part of the Gaussian software package.³⁴ Partial charges on the head group of the DiI molecule were obtained by performing *ab-initio* quantum mechanical calculations using Gaussian03 with the Hartree–Fock method and the 6-31G basis set using the charge partitioning scheme of

Merz–Kollman (MK method)³¹ (see Table 1 and Fig. 2 for the distribution of partial charges on the chromophore). The DiI head group contains a final charge of +1 and the existence of the partially polarized double bond between N19 and C29 may introduce some asymmetry in the charge distribution. To ensure electrical neutrality of the system, 2 or 4 Cl⁻ ions (for 2-dye and 4-dye bilayers, respectively) were added to the system at random positions. Alkyl chain carbon atoms on DiI were assigned a charge of zero similar to the acyl chains of the DPPC molecules. For water, the simple point-charge model (SPC) was used for all simulations.³⁵

The molecular dynamics simulations were carried out using the GROMACS simulation software package (version 3.2).³⁶ Energy minimization was performed to remove any unfavorable contacts resulting from the model building procedure. Pre-equilibration of the energy-minimized structure was performed using conditions of constant number, volume, and temperature (NVT), with the temperature set at 323 K. The total runtime for the pre-equilibration was 10 ns for each of the simulations. After pre-equilibration, the structure was energy minimized and the production runs were commenced.

MD simulations were carried out under conditions of constant number, pressure and temperature (NPT). Periodic boundary conditions were applied to the simulation box in all three coordinate directions (with x , y in the bilayer plane, and z normal to the bilayer). The simulation temperature was 323 K which is above the gel-to-liquid phase transition temperature, T_M , of DPPC $\approx$ 315 K. The temperature and pressure of the system were controlled by using the Berendsen algorithm with the time constants set to 0.1 and 1.0 ps, respectively.³⁷ Anisotropic scaling was applied to the simulation box with the diagonal components set to zero, ensuring the box was free to expand independently in the 3 coordinate directions. The time step was 2.0 fs and the coordinates from the simulation were saved once every 10 ps. The LINCS algorithm was used to constrain the bond lengths.³⁸ For each of the DiI simulations, (0-dye, 2-dye, 4-dye, and no charge) the particle-mesh Ewald (PME) electrostatics scheme was applied. A cutoff for 1 nm was applied in the direct-space sum for short-range interactions. Cubic interpolation was used to project the charges onto a grid for calculation of long range interactions in reciprocal space. The maximum spacing of the Fourier transform of the grid used in the simulation was 0.12 nm. The Lennard-Jones interactions were cutoff at 1.0 nm uniformly. The total duration of each simulation was 40 ns. A snapshot of the 4-dye simulation is shown in Fig. 1. The last 35 ns of simulation time were considered for analysis of all quantities unless otherwise stated.

Results and discussion

Data analysis was conducted to (i) determine the perturbative influence of DiI-C₁₈(3) on the physical and structural properties of DPPC bilayer, (ii) determine the axial location and orientation of DiI, (iii) determine effects of DiI on interfacial bilayer properties, and (iv) assess the fidelity of DiI in representing DPPC molecular dynamics. In each case, MD data is compared with available experimental data, in order to assess reliability of the simulation, and to provide insight not accessible with current experimental techniques.

Influence of the dye on the physical and structural properties of the DPPC bilayer

DiI has negligible effect on area per phospholipid—The average area of lipid is related to membrane compressibility, molecular packing, and acyl chain ordering^{39,40} and is reflected by measurements of lateral diffusion, rotational diffusion, and bilayer permeation. The time-dependent area per lipid is also a good indicator of simulation stability. The area per lipid was computed by dividing the instantaneous size of a lipid bilayer by the number of molecules present per bilayer (=64) in the x - y plane. The areas per lipid resulting from the simulations are shown in Fig. 3.

The areas per lipid obtained for 4 simulations (0-dye, 2-dye, 4-dye, and no charge) were $\langle A \rangle = 0.637 \text{ nm}^2$, $\langle A \rangle = 0.647 \text{ nm}^2$, $\langle A \rangle = 0.640 \text{ nm}^2$, and $\langle A \rangle = 0.637 \text{ nm}^2$, respectively. The values obtained for the case when there are no DiI molecules present in the bilayer (0-dye) compare favorably with experimental values of area per lipid which average 0.64 nm^2 for DPPC.⁴¹ However, the experimental value of the area per lipid can vary from 0.56 to 0.71 nm^2 .⁴¹ Thus, we conclude that DiI : lipid ratios of 2 : 126 (2-dye) and 4 : 124 (4-dye and no charge) do not significantly affect the average area per lipid of the DPPC bilayer. Furthermore, equilibration of area per lipid within the first 5 ns demonstrates simulation stability.³⁹

DiI induces an increase in membrane thickness—We computed the thickness of the bilayer as a function of DiI concentration from the distance measured between the two crossover points for density values of the lipid and water²⁸ (see Fig. 5). The thickness of the bilayer was 4.01, 4.22, 4.15 and 4.20 nm for 0-dye, 2-dye, 4-dye, and no charge, respectively. These absolute thickness changes correspond to increases of 5.2% (2-dye), 3.4% (4-dye) and 4.7% (no charge) relative to the 0-dye case. Thus, we observe that DiI-C₁₈(3) increases bilayer thickness but these increases are not linear with increasing DiI concentration (2-dye *versus* 4-dye). In addition, the mass density of the atoms in the center of the bilayer fell from 638 kg m^{-3} in pure DPPC bilayer to 546, 520 and 507 kg m^{-3} for 2-dye, 4-dye, and no-charge cases, respectively. The decrease was maximal in the no-charge case, possibly due to localization of uncharged DiI in the bilayer center (see Fig. 5B). Thus, the positively-charged DiI-C₁₈(3) chromophore reduces DPPC acyl chain density leading to increases in bilayer thickness.

Although area per lipid predictions do not suggest DiI-perturbation, DiI-induced thickness changes may be significant from a physiological point of view. For example, decreases in membrane thickness of $\sim 0.1 \text{ nm}$ are associated with bilayer thickness changes of 2–4% which may be sufficient to gate MscL mechanosensitive ion channels (5) suggesting that increases in membrane thickness on this order may affect integral membrane protein conformation and function. Therefore, our simulation results suggest that high concentrations of DiI may lead to significant and physiologically-relevant membrane perturbation. Since FCS uses concentrations that are 100 fold lower than the simulated concentrations it is unclear whether FCS-level concentrations appreciably affect membrane thickness.

DiI increases order in bilayer interior—The effect of DiI-C₁₈(3) on the packing of the lipid bilayer interior was deduced by computing the changes in the deuterium order parameter, S_{CD} , along the lipid chain. The order parameter is a quantity which can be obtained from experiment using deuterium NMR. S_{CD} is calculated using the equation,

$$S_{\text{CD}} = \langle \frac{3}{2}(\cos^2 \theta_{\text{CD}}) - \frac{1}{2} \rangle \quad (1)$$

where θ_{CD} is the time-dependent angle between a C–H bond along the alkyl or acyl chain and the membrane normal (z -axis) and the brackets denote a time and ensemble average. Due to the use of a united atom description for the carbon atoms, the position of three successive CH₂ groups was used to reconstruct the CH bond, assuming tetrahedral geometry of CH₂ groups. The value of S_{CD} can vary from -0.5 , indicating full ordering along the plane of the bilayer, to 1, corresponding to full ordering along the normal to the bilayer. The order parameters of the DPPC sn-1 and sn-2 acyl chains are shown in Fig. 4. The computed values of S_{CD} for pure DPPC bilayer (0-dye) show good agreement with the values obtained *via* experiment and previous simulations.^{5,42,43}

In the presence of DiI (2-dye and 4-dye), there was an increase in the S_{CD} parameter along the length of the carbon sn-1 and sn-2 chains (Fig. 4A and 4B). In the case of sn-1 chains, the increase in the ordering was present near the terminal carbon atoms (Carbon # ≥ 5) and was uniformly increased throughout the length of the carbon atom chain (see Fig. 4A). The maximal increase in the sn-1 chain ordering occurred for the no-charge simulation probably due to the fact that localization of the no-charge dye chromophore to the center of the bilayer resulted in an ordering effect.

Similar to sn-1 chains, DiI-C₁₈(3) inclusion resulted in a slight increase in the order parameter of carbon atoms of the sn-2 acyl chains. However, the magnitude of the increase in the order parameter was maximal in the initial (C1–C8) carbon atoms, though the increase in the order parameter was observed throughout the length of the chain (see Fig. 6B). The maximal increase in the ordering was in the case of the 4-dye simulation unlike the sn-1 chains where the maximal ordering was observed for the no-charge case. One possible explanation for the difference of ordering between the sn-1 and sn-2 chains is that the sn-2 chains are located on average closer to the lipid–water interface compared to the sn-1 chains. The mass density profiles (see Fig. 5) suggest that the charged DiIs (2-dye and 4-dye) were located near the interface and were thus likely to influence the sn-2 chain of the DPPC molecule more than the sn-1 chain. On the other hand, localization of the no-charge DiI molecules towards the center is likely to affect the order parameters of the sn-1 chain. More importantly, the presence of DiI did not destabilize the native lipid bilayer structure as indicated by lack of change in bilayer order. On the contrary, the presence of DiI led to a slight increase in the average ordering properties of the bilayer. A similar effect of increased ordering in the fluid phase of a DPPC bilayer was noted for fluorescent pyrene molecules in a related study.²⁹

Location and orientation of the dye molecule in the bilayer

DiI chromophore resides just below lipid head groups—Interpretation of experimental fluorescence measurements from rotational anisotropy, fluorescence lifetime, and fluorescence quenching depends on knowledge of the location of the dye molecule within the bilayer.²⁵ The location of DiI could potentially be determined using X-ray crystallography or NMR studies or using indirect methods such as fluorescence quenching. However, no structural studies (X-ray crystallography or NMR) have been reported on lipid bilayers containing DiI. Thus the present MD simulations provide the first detailed prediction of DiI location in a lipid bilayer.

In order to determine the precise predicted DiI location, we plotted the mass density profile of various components of water, DiI-C₁₈(3), and DPPC molecules. To eliminate effects due to fluctuations of the center of mass (COM) of the bilayer with time, the position of COM of the whole bilayer in each frame was subtracted from mass density positions at each instant. The location $z = 0$ in Fig. 5 corresponds to the center of the bilayer.

In addition, we plotted the axial location of the DiI chromophore and the whole DiI COM positions along with the axial positions of the DPPC phosphorus and nitrogen atoms. The phosphorus and nitrogen atoms are part of the lipid–water interface and provide a reference plane with which to compare the location of DiI. The spread of the density profiles of the DiI chromophore and the whole DiI molecule exhibited a range of axial (z) locations (see Fig. 5B, C, D). More importantly, the average peak-to-peak density distance between the phosphorus atoms in the DPPC molecules and DiI chromophore was 0.63 nm (values averaged over all 6 DiI molecules) and ranged from 0.3 to 2 nm from the DPPC-head group–water interface. In the no-charge simulation, the DiI chromophore was located 1 nm below the peak phosphorus density. Further analysis of the time-dependence of DiI location showed that the chromophore COM remained in one leaflet at a position averaging 1.26 nm

from the center of the bilayer and ranging from $z = 0.3$ nm to $z = 2$ nm (see Fig. 6A). We conclude that the DiI chromophore is situated below the lipid–water interface and with water accessibility to the chromophore determined from DPPC head group packing. We also conclude that the relatively long carbon tails of DiI are permitted, energetically, to cross the center of the bilayer and extend into the opposite leaflet. Stable location of DiI in one leaflet is dependent on the chromophore's positive charge, as non-charged DiI tended to flip-flop to the opposite leaflet (Fig. 6B).

The mass density profile of the 0-dye case compares well to previous studies of molecular dynamics of DPPC bilayers,^{28,44,45} suggesting that the simulation results are robust. The conclusion that the DiI chromophore resides below the DPPC head group–water interface is in contrast to many cartoon representations of the DiI location. However, the result is similar to experimental results by Krishna *et al.* who concluded from fluorescence lifetime and anisotropy data that a dye with a similar headgroup can exist below the head group–water interface.^{27,46} Second, charge plays an important role in determining the location of the dye as seen by localization of the DiI near the hydrophobic center of the bilayer in the absence of any charges on the chromophore (no-charge). Thus, our results suggest that MD simulations are a viable method to determine precise DiI locations in lipid bilayers and yield insight into membrane structure not currently accessible by experimentation. Our results are also consistent with the observed enhancement of fluorescence of DiI upon incorporation into membranes. In addition, the model for DiI in which the chromophore is above the lipid–water interface is energetically unfavorable since it would expose the non-polar hydrophobic acyl chains of DiI to the aqueous solvent. Most importantly, our simulations support the notion that sufficient free volume exists in the region just under the water–head group interface to support fast anisotropy components and wobbling in a cone, as proposed by Krishna.²⁷ To support this conclusion, Lopes and Castanho inferred the location of a structurally similar kind of dye, oxycarbocyanine, to be present in the interfacial region based on fluorescence anisotropy studies.⁴⁷

When bilayers containing uncharged DiI were simulated (no-charge), the chromophore region of two of the DiI molecules was observed to intercalate into the opposite leaflet of the bilayer constituting what is known as a “flip-flop.” In previous experimental studies by Wolf, *et al.*,¹⁶ carbon DiIs were used to study the *trans*-leaflet migration of DiI in egg phosphatidylcholine vesicles.²⁴ Trinitrobenzenesulfonate (TNBS) was used as a non-permeating quenching agent of the bilayers to study the inner leaflet transmigration. It was concluded that the flip-flop process of DiI was an extremely rare event, occurring on time scales of no shorter than 4 h at temperatures between 4 and 37 °C.²⁴ Thus, we conclude that the positive charge on DiI stabilizes the localization of the headgroup to just below the lipid–water interface and confines it to a single leaflet.

The long axis of DiI chromophore is nearly parallel to DPPC bilayer plane—

Correct interpretation of fluorescence polarization and anisotropy depends on knowledge of the location and orientation of the DiI chromophore moiety.²⁵ To assess the orientation of DiI-C₁₈ (3) chromophore relative to the membrane–water interface, we computed the angular distribution of the chromophore with respect to the lipid bilayer normal (along the z -axis). A chromophore orientation vector was defined from the center of mass position of the phenyl ring (atoms 20–25, see Fig. 2) at one end of the chromophore to the center of mass position of the opposing phenyl ring (atoms 37–42). The coordinates and direction of this vector were monitored during the course of the simulation. The orientation angle, θ (not to be confused with θ_{CD}), is defined as the angle made by the vector and the bilayer normal. An orientation angle of 0 or 180° corresponds to the chromophore lying parallel to the bilayer normal whereas an angle of 90° corresponds to the chromophore lying perpendicular to the lipid bilayer normal and parallel to the lipid–water interface.

Normalization of the number density of the angular distribution over the entire course of the simulation resulted in the probability distribution function shown in Fig. 7A and B. Fig. 7A represents the results obtained for such a probability distribution function in the case where the chromophore groups were assigned a charge of +1 (2-dye and 4-dye). Fig. 7B represents the case where no charge is assigned to the chromophore head group (no-charge). For positively-charged dyes, the orientation angles of the chromophore were distributed over a narrow range. The distribution is plotted for all 6 DiI molecules (2-dye and 4-dye). The distribution was not uniform and was multimodal in nature (except for one dye). The mean orientation of the distribution was $\theta = 77^\circ$. Thus, the presence of a positive charge on the chromophore results in an orientation of the dye chromophore which is nearly parallel to the plane of the bilayer. However, the spread of the angular distribution indicates that the chromophore samples a range of orientations from 30 to 122° with a full-width at half maximum (FWHM) of $\Delta\theta = \pm 17^\circ$.

Fluorescence polarization microscopy has been used to study the orientation of DiI in artificial planar membranes^{48,49} and red blood cells.^{50,51} Consistent with our simulation results, the transition dipole moment of DiI lies parallel to the lipid–water interface.^{48–50} Axelrod and coworkers studied the orientation of DiI-C₁₈(3) in red cell ghosts and proposed a model for the orientation of the dye which accommodated $\pm 10^\circ$ of parallelism to the lipid–water interface by adjustment of the experimental fits of the model.⁵⁰ Considering the limited number of DiI molecules and short timescales used in the simulation, the experimental ranges correspond well to our simulation results.

In the absence of a chromophore charge, the mean angle of orientation averaged over 4 DiI molecules was $\theta = 87^\circ$. The FWHM of the angular distribution was $\Delta\theta = \pm 33^\circ$ reflecting a much broader distribution of angles. The distribution extended from 12 – 174° . The distribution was multimodal similar to the case of charged DiI. However, the wide range of angle distribution is a reflection of DiI tumbling permitted because of the absence of charge on the chromophore (see previous section). The multimodal distribution indicates the presence of localized restrictive potential of the surrounding DPPC molecules leading to intercalation of the DiI chromophore between the DPPC acyl chains.

In order to gain a further understanding of the relationship between the location of DiI and orientation angle of the DiI chromophore, we plotted the angle of the orientation of the chromophore against the location of the center of mass of the chromophore. In the case of the positively-charged DiI molecules, there was a distinct correlation between DiI-C₁₈(3) location and angle of orientation (see Fig. 8A) in which head groups located between 1.0 – 1.4 nm from the center of the bilayer along the z -axis exhibited angular orientations ranging from 60 – 120° . In the absence of a positive charge on the chromophores (no-charge), all angles of orientation were possible and the majority of the angles occurred between 0.4 – 1 nm from the center of the bilayer (see Fig. 8B). Thus we can conclude that the positive charge on the chromophore results in constrained angular motion of the chromophore by virtue of the positive charge association with the base of the lipid head groups.

Effects of the dye on the structural properties of the lipid–water interface

DiIs have been used to study changes in membrane potential by measuring the changes in the fluorescence emission.⁵² The precise mechanisms behind changes in fluorescence emission are complicated and not well understood.^{52,53} While it is not currently feasible to compute fluorescence emission properties, which are based on quantum mechanics, it is of interest to understand changes in the interfacial structural properties due to the presence of DiI in the lipid–water bilayer interface because these properties may be related to experimentally-measurable fluorescence properties such as fluorescence life-time and intensity, which are related to membrane potential. Thus, we computed orientation of the

water dipole, electrostatic potential across the interface, and DPPC P–N vector orientation in the presence and absence of DiI.

DiI induces shift in ordering of water dipole—The ordering of the water dipole was computed by calculating the time-averaged projection of the water-dipole unit vector $\hat{\mu}(z)$ onto the interfacial normal (z -axis). At the DPPC–water interface, the water dipoles pointed towards the bilayer center as seen by the minima of the value of the average cosine angle of the water dipole vector (see Fig. 9A). Away from the interface, the ordering persisted in the z -axis until the point where the lipid density approached zero. The minimum also corresponded to the peak value of the density of the phosphorus atoms at the lipid–water interface as shown in previous studies.³⁹ In the current sets of simulations we compared the distance between the minima value of the time-averaged dipole vector of water in the presence and absence of DiI. The maximal orientation of the water dipole was located at $z = 1.76$ nm for the 0-dye case (Fig. 9). This value corresponds well to the data for a pure DPPC bilayer.³⁹ In the presence of the dye molecules the location of the minima shifted to $z = 1.82$ nm, $z = 1.84$ nm and $z = 1.83$ nm for the 2-dye, 4-dye, and no-charge cases, respectively. Thus the presence of DiI-C₁₈(3) led to a minor but significant effect on the ordering of water dipoles across the lipid–water interface which corresponds to the DiI-induced increase in bilayer thickness. Thus we can conclude that addition of DiI-C₁₈(3) leads to ordering of water at the interfacial region mainly as a consequence of increase in the thickness of the bilayer (see the mass density profile section).

DiI affects electrostatic potential across the membrane—Electrostatic potential profile at the lipid–water interface is the result of the interplay between the ordering of water dipoles and the distribution of the lipid dipoles. To determine electrostatic potential, the simulation box was divided into slices along the z -axis and the time-averaged charge density was computed for each individual slice. The electrostatic potential $V(z)$ across the lipid bilayer was then computed by integrating the charge density twice using Poisson's equation.

$$V(z) = \psi(z) - \psi(0) = -\frac{1}{\epsilon_0} \int_0^z dz' \int_0^{z'} \rho(z'') dz'' \quad (2)$$

where $\psi(z)$ and $\rho(z)$ are the time-averaged dipole potential and the charge density, respectively, as a function of the bilayer normal. ϵ_0 is the permittivity of vacuum. The potential was computed with respect to the center of the bilayer. The resulting potential profile is shown in Fig. 9B. In the case of the pure DPPC bilayer (0-dye) the potential difference, ΔV , between the center of the bilayer and bulk water was -550 mV. This value is similar to the values obtained from previous simulations of pure DPPC bilayers.^{40,42} The lipid molecules contribute with a positive potential which was then slightly overcompensated for by the negative potential of the water dipoles. Experimentally, the values for the potential difference vary from -200 to -575 mV for various phosphocholine–water interfaces.

The presence of DiI led to a shift in the potential difference across the lipid–water interface. In the case of 2 DiI molecules (2-dye), ΔV was -636 mV. Upon addition of 4 DiI molecules (4-dye) the potential difference was -692 mV. More interestingly, the electrostatic potential profile for the no-charge case was similar to the pure DPPC case. The potential difference, ΔV , in the no-charge case was -556 mV (similar to -552 mV for 0-dye).

We note that the electrostatic potential profiles are correlated with the mass density profiles (see Fig. 5) and the orientation of water dipoles (see Fig. 9A). The differences in the packing

of the lipids at the interface are reflected as changes in the electrostatic potential profiles. Significant rearrangements of the lipid head group dipole moment due to presence of the charged DiI may be the cause of differences in the electrostatic potential profiles across the lipid–water interface. One way to analyze the rearrangements in the lipid dipole is to compute the orientational distribution of the P–N angle of the DPPC head group, as is done in the next section.

DiI influences lipid head group orientation: analysis of P–N vectors—To study the reordering of lipid dipoles and the packing of the lipids head groups at the interface in the presence of positively charged DiI, we computed the angular distribution of the P–N vector of the DPPC lipid molecules. The P–N vector is defined by a line drawn from the phosphorus (P8) atom to the nitrogen (N4) atom in the DPPC head group. An angle of 0° corresponds to the vector aligned parallel to the bilayer normal, while an angle of 180° corresponds to the vector pointing straight into the bilayer core. Fig. 10 illustrates the normalized distribution of P–N vector angles computed over the entire duration of simulation. In the absence of DiI, the peak of the distribution of the vector was 90° . Thus the orientation of the P–N vector in a pure DPPC bilayer is approximately parallel to the lipid–water interface. The distribution was monomodal with the full-width at half maximum (FWHM) of $\Delta\theta = 70^\circ$. The values of the P–N vector distribution for the 0-dye case are similar to results seen in previous simulations.

In the presence of two positively-charged DiI molecules (2-dye), the peak of the P–N vector distribution shifted by 6° to a value of 84° pointing towards the aqueous phase. The FWHM of the 2-dye distribution increased to $\Delta\theta = 73^\circ$. In the case of the four positively-charged dye molecules (4-dye), the peak of the distribution was shifted further by 7° to 77° towards the aqueous phase. The FWHM decreased further to $\Delta\theta = 71^\circ$ reflecting a narrower distribution. In the absence of charge on DiI (no-charge), the peak of the P–N vector distribution was 91° , which is similar to the value of a pure DPPC bilayer (90°). The FWHM of the distribution was slightly less compared to 0-dye with a value of $\Delta\theta = 68^\circ$. Thus, we conclude that the presence of positive charges on the DiI molecule leads to a reordering of the lipid dipoles across the interface. The shift of the P–N vector into the aqueous interface in the presence of positively-charged DiI is probably a result of subtle, localized electrostatic interactions between the charged dye and the lipid dipoles. Recalling that uncharged DiI did not lead to a change in bilayer thickness, an increase in the bilayer thickness is expected to play only a minor role in the perturbation of the lipid dipole order across the interface as seen by the absence of any shift of the P–N vector in the absence of a charge on DiI.

DiI and DPPC dynamics

Spatially-varying lateral diffusion of lipids in both model and native cell membranes can be measured using FCS and FRAP.^{12,54} Such measurements of alteration in the lateral diffusion of lipids are important because changes in lipid diffusion may initiate or accompany changes in integral membrane protein conformation and association leading to changes in signal transduction (*e.g.*, phosphorylation and dimerization). While FCS and FRAP yield data on dynamics that occur over time scales of microseconds to seconds, nuclear magnetic resonance (NMR) can provide data on much faster rates of diffusion due to the short measurement time-scales. Molecular dynamics probes events occurring on time scales intermediate between NMR and FCS, thus providing a means to verify the validity of experimental results. To test the fidelity with which DiI dynamics reflects DPPC dynamics, we examined rotational and lateral diffusion of DiI and DPPC molecules as determined from simulation and compare these values to experimental values reported in the literature.

Rotational dynamics of DiI and DPPC are comparable—Understanding the rotational dynamics of the chromophore may yield improved interpretation of fluorescence anisotropy. The fluorescence anisotropy decay is influenced by various factors such as the rotational dynamics of the fluorophore, the angle between the absorption and emission dipoles, and the surrounding environment of the fluorophore.²⁵ We computed the rotational correlation function in order to understand the rotational dynamics of DiI-C₁₈(3). The rotational correlation function, $C(t)$, is defined as

$$C(t) \equiv \langle P_2(\hat{\mu}(0)\hat{\mu}(t)) \rangle = \langle P_2(\cos\theta(\eta)) \rangle \\ = \langle \frac{3}{2}(\hat{\mu}(0)\hat{\mu}(t))^2 - \frac{1}{2} \rangle \quad (3)$$

where $\hat{\mu}(0)$ and $\hat{\mu}(t)$ are unit vectors separated by time t and $\theta(\eta)$ is the angle between the vectors. The brackets signify the average over all time and $P_2(x)$ is the second Legendre polynomial given by:

$$P_2(x) = \frac{3}{2}x^2 - \frac{1}{2} \quad (4)$$

The transition dipole vector of the fluorophore is the axis along which the absorption of the photons takes place. For DiI-C₁₈(3), this axis has been proposed to lie along the conjugated bridge connecting the two indole rings.^{48,50} We define an axis connecting the atoms C27 to C33 as the axis along which the transition moment of the fluorophore molecule is present. This axis was used to compute the rotational correlation function. Experimentally, the fluorescence anisotropy decay of an isotropic dipole distribution is given by:

$$r(t) = 0.4 \langle P_2(\hat{\mu}_a(\eta)\hat{\mu}_a(t+\eta)) \rangle \quad (5)$$

where $\hat{\mu}_a$ and $\hat{\mu}_e$ are the absorption and emission vectors of the transition dipole moment of the fluorophore. The above equation is similar to the rotational correlation function except for the 2/5 scaling factor in the anisotropy.⁵⁵ Thus the timescales of decay in both equations are of comparable order.

The rotational correlation function, $C(t)$ of the transition moment vector averaged over 6 positively-charged DiI molecules is shown in Fig. 11. Results show that rotational correlation function did not decay to zero indicating that the rotational correlation function did not reach its asymptotic limit. This result is a possible consequence of the relatively short duration of the simulation. However, incomplete decay of the fluorescence anisotropy is also routinely observed experimentally. A double exponential function is commonly used to fit experimental anisotropy data. We used a similar equation (eqn (6)) to fit the curve shown in Fig. 11:

$$C(t) = C_0(\beta_1 \exp(-t/\tau_1) + \beta_2 \exp(-t/\tau_2)) + C_\infty \quad (6)$$

The important values obtained from this fit are the short and long timescales, τ_1 and τ_2 , whose probable origins are the rotational motion of the chromophore and the whole dye molecule, respectively. Results of $\tau_1 = 0.99$ ns and $\tau_2 = 6.9$ ns represent the fast and slow components, respectively, of the rotational correlation function. For comparison, Ariola *et al.* experimentally determined the anisotropy decay of a structurally related dye, DiI-C₁₂(3), in a fluid phase bilayer of DOPC and obtained values of $\tau_1 = 1.2$ ns and $\tau_2 = 9.6$ ns, respectively.⁵⁶ Krishna *et al.* studied the fluorescence decay of DiI-C₁₈(5) (longer

conjugated bridge compared to DiI-C₁₈(3)) and obtained a value of $\tau_1 = 0.18$ ns and $\tau_2 = 1.6$ ns, respectively.²⁷ Thus, the values for the anisotropy decay time-scales obtained from the experimental data compare favorably with the rotational correlation data of the simulation. Although there are differences between the methods used to measure anisotropy between the two studies that may lead to some interesting insight into the origins of the fast and slow components, an important point is that the location of the chromophore, as determined from the simulation, is compatible with the origin of the fast component of the fluorescence decay of DiI-C₁₈(3). Thus, steric hindrance of the chromophore by the base of the lipid acyl chains (just under the head group) is not sufficient to suppress the fast component. In other words, it is not necessary to interpret fast components of anisotropy as evidence that the chromophore is in the aqueous phase of the bilayer.

Lateral diffusion of DiI and DPPC are comparable—Lateral diffusion of lipid molecules is an indicator of lipid viscosity, which governs many membrane-related cell signaling processes.^{15,50} To quantify the lateral dynamics of DPPC and DiI, we computed the mean square displacement (MSD) of the COM of the lipid and the dye as a function of time. The diffusion coefficient, D , is given by the slope of the average mean square displacement at long times. More specifically, the lateral diffusion coefficient, D , is defined through the Einstein relation,

$$D = \lim_{t \rightarrow \infty} \frac{1}{2dt} \langle [\vec{r}_i(t+t') - \vec{r}_i(t')]^2 \rangle \quad (7)$$

where $\vec{r}_i$ is the x, y position of the center of mass of a lipid i at any given time, t , and d is the dimension used in the calculation. Brackets denote the average over all time t' . In the case of bilayers, d is equal to 2. The diffusion coefficient was computed over all the molecules in the bilayer and averaged. It was important to keep in mind that the lipid bilayer leaflets move relative to each other during the simulation even though the motion of the center of mass position is constant. This relative motion can lead to apparent artificial supra-diffusive motion of the individual molecules in the bilayer. This potential artifact was taken into account by removing the COM motion of each leaflet of the bilayer relative to the system motion. The lateral diffusion coefficient thus calculated corresponds to the motion of each individual lipid molecule in the absence of lipid flow.

In our simulation, we compared the values of the diffusion coefficient for the DPPC and DiI-C₁₈(3) molecules separately (see Fig. 12). The value of the diffusion coefficient of DPPC was $10.7 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ for a 1.1 nm^2 displacement over the simulation time. The value of the diffusion coefficient of DiI-C₁₈(3) was $9.7 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$, corresponding to a 1.01 nm^2 displacement over the duration of the simulation. Diffusion in DPPC bilayers has been measured by FRAP to be $12.5 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ at 325 K, which is above the phase transition temperature of 314 K.⁵⁷ Using quasi-electric neutron scattering, a value for diffusion coefficient of $15 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ was obtained at a temperature of 333 K.⁵⁸ Experimentally, Kahya *et al.* obtained values of diffusion of DiI-C₁₈(3) in a structurally similar lipid matrix composed of DOPC. D obtained in that study was $6.3 \pm 0.2 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$.¹³ Thus, the values obtained from simulation correspond well to those obtained from experiment. Importantly, DiI diffusion closely matched DPPC diffusion.

In the majority of experimental studies that measure lipid diffusion, the data collected correspond to the actual diffusion rates of the probe molecule and not that of the native lipid itself. The DiI diffusion rates are considered to be a reflection of the native lipid rates. In this study we show that the correspondence between the lipid and DiI molecular diffusion rates

is close at long times. Thus we have established that DiI lateral dynamics are representative of the lipid molecules making up the bilayer.

Conclusions

The main findings of this study are as follows: DiI has minimal effect on area per lipid but may affect membrane thickness. Second, DiI axial location in DPPC is below the head group–water interface and steric hindrance below the interface is not sufficient to suppress a fast anisotropy component. Third, DiI induces a change of lipid and water physical properties near the headgroup–water interface. Fourth, DiI reflects well the rotational and lateral diffusive components of lipid dynamics.

An issue of major concern in experimental fluorescence studies is whether the dye perturbs the physical properties of the native lipid bilayer. We explored this issue by measuring various physical properties of the bilayer in the presence of increasing concentrations of DiI-C₁₈(3). In spite of relatively high concentrations of dye used (1.6 mol% (2-dye) and 3.2 mol % (4-dye)), we observed only a minor perturbation of the bilayer physical properties as represented by area per lipid, and order parameters of the DPPC acyl carbon tails. However, bilayer thickness may be affected appreciably by inclusion of these high concentrations of DiI. Current lack of NMR structural data on mixtures of DiI and DPPC precludes any direct comparison of the simulation data to experiment. Such data would be useful in validation of the MD simulation and represents a potential area of investigation.

Regarding DiI location, previous models of DiI generally placed the dye chromophore at an exterior location in the aqueous phase.⁴⁶ Krishna *et al.* have shown that DiI location may depend on subtle properties of the bilayer such as saturation state and fluidity. Consistent with these results, we show that DiI is located well below the interface, below the phosphorus and nitrogen atoms of DPPC molecules, which exist in the fluid state at the simulation temperature. The DiI chromophore does not cross the interface at any point during the simulation. This result is also consistent with the known enhancement of DiI fluorescence when incorporated into bilayers. In addition, the positively-charged chromophore plays a role in the localization of the DiI as seen by the “tumbling” motion undergone by the dye in the absence of a charge. We also note that the positive charge on the chromophore governs DiI’s orientational distribution and yields a result consistent with experimental data using polarization microscopy⁵⁰ thus validating the use of molecular dynamics studies to determine rotational dynamics of fluorophores in bilayers.

DiIs have been used extensively as fluorescent indicators of membrane potential.⁵² While it is difficult to conclude from MD simulations the direct relationship between fluorescence and membrane potential, we were interested in assessing possible effects of the dye on interfacial properties, which include membrane potential. We observe that DiI-C₁₈(3) causes at the interface minor changes in the ordering of the water dipoles, electrostatic potential, and an altered structure of the lipid dipoles. The changes in the properties of the lipid–water interface were a result of the presence of the positively-charged dye near the interface. In the absence of charge, the perturbation at the lipid–water interface was minimal and similar to the case where DiI molecules were absent.

Finally, we examined rotational and lateral dynamics of DiI-C₁₈(3). The rotational correlation function of the transitional moment vector of the fluorophore exhibited a timescale of rotation which was similar to structurally-comparable DiIs such as DiI-C₁₂(3) and DiI-C₁₈(5). More importantly, the chromophore displayed rotational dynamics comparable to experimental anisotropy values despite its predicted location inside the lipid bilayer. We conclude that the fast component of the anisotropy decay can be explained

without the need of localizing the chromophore above the lipid–water interface. DiI-C₁₈(3) has been assumed to represent the structural and dynamic properties of the lipid bilayer much more closely compared to structurally dissimilar fluorescent probes. We computed the lateral diffusion of DiI-C₁₈(3) and compared it to the lateral diffusion of DPPC molecules and found them to be comparable. Thus, we conclude that DiI is an excellent reporter probe of lipid dynamics and may be a sensitive reporter of surface phenomena occurring at the bilayer interface.

The use of DiI is pervasive in membrane studies and correct interpretation of experimental fluorescence data depends on the detailed knowledge of the location and orientation of the fluorescent probe. In this paper we provide atomistic level molecular dynamics simulations of DiI in a model membrane to determine DiI effects on membranes as well as atomistic insight into DiI location, dynamics, and interfacial phenomena. Such studies provide data on the physical and dynamic properties of positively-charged DiI-C₁₈(3) in a fluid phase membrane of DPPC at 325 K. This approach has been used successfully to investigate the properties of other fluorescent dyes such as DPH,²⁸ pyrene-PC,²⁹ free pyrene⁵⁹ and NBD-PC.³⁰ Continuing use of MD to study fluorescence probes opens up new avenues to interpret the wealth of information available from time-resolved fluorescence spectroscopy and single-molecule fluorescence studies. Combining these computational and experimental approaches will provide highly accurate inferences of the molecular origins of bilayer structure and function of native systems from experimental observables.

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Fig. 1.

A snapshot of the simulated bilayer containing four DiI molecules and 124 DPPC molecules and water. This DiI concentration corresponds to a final concentration of 3.2 mol%. The other concentration used in the simulation is 1.8 mol% (2-dye). The DiI molecules are located below the lipid–water interface with their chromophore long axes oriented parallel to the lipid–membrane interface.

Fig. 2. Schematic representations of molecules used with atoms numbered. The partial charges used on the DPPC molecules are also shown. The acyl and alkyl carbon atoms are assigned a partial charge of zero for DPPC and DiI-C₁₈(3) molecules, respectively. For a description of the partial charges on the DiI-C₁₈(3) molecule, refer to Table 1.

Fig. 3.

Area per lipid. The average values of the area per lipid are indicated as a function of DiI concentration and simulation time (last 35 ns).

Fig. 4.

The deuterium order parameters (S_{CD}) *versus* the chain position for the sn-1 and sn-2 acyl chains of DPPC molecules. The order parameters shown are in the presence (2 and 4 DiI molecules, and 4 uncharged DiI molecules) and absence of DiI (0-dye).

Fig. 5. Mass density profiles across the lipid bilayer of four simulations, (A) 0-dye, (B) no charge, (C) 2-dye, (D) 4-dye. The density was averaged over the last 35 ns of the simulation. The individual profiles of various components, namely, DPPC, water, DiI-dye, DiI head region, phosphorus atoms and nitrogen atoms of DPPC are shown. The density profile of the DiI head and whole DiI was scaled by a factor of 10 for purposes of clarity. Similarly, the density of P and N atoms is scaled by a factor of 3. The thickness of the bilayer is indicated by the distance between the crossover region of DPPC and water density marked by circles in A.

Fig. 6.

Location of DiI along the z-axis during the simulation. (A) The trajectory of a positively charged DiI present in each leaflet of the bilayer is shown. The plot shows the trajectory of the head group of DiI, center of mass position of DiI, center of mass position of the whole bilayer and the position of phosphorus and nitrogen atoms of DPPC for reference purposes. (B) Similar to A, but the trajectory of an uncharged DiI is shown.

Fig. 7.

Normalized distribution of angular orientation of the DiI head group with respect to the lipid bilayer interface. The chromophore orientation vector is defined from COM position of phenyl ring (atoms 20–25, see Fig. 2) to the COM position of the opposing phenyl ring (atoms 37–42). (A) Orientations adopted by positively charged DiI molecules (2-dye and 4-dye). (B) Orientations adopted by DiI in the absence of charge (no-charge). A value of 90° indicates the DiI chromophore long axis is oriented parallel to the lipid–water interface. Each symbol represents a different DiI molecule.

Fig. 8. Angular distribution of the head group of DiI as a function of the z -location in the DPPC bilayer. (A) Distribution in charged DiI. (B) Distribution in uncharged DiI.

Fig. 9.

(A) Ordering of water molecules across the lipid–water interface in DPPC bilayer. The $\langle \cos \theta \rangle$ is the cosine of the angle of the water dipole vector with respect to the normal of the lipid bilayer. A value of zero corresponds to a completely disordered state. The minimum of the curve corresponds to location of maximal ordering of water molecules. (B) The electrostatic potential ($V(z)$) across the lipid bilayer. $z=0$ is the location of the center of the bilayer in both panels.

Fig. 10.

The angular distribution of the DPPC P-N vector with respect to the normal of the lipid bilayer. The vector is defined as a line connecting the phosphorus atom (atom 8) and the nitrogen atom (atom 4) in a DPPC molecule (see Fig. 1). The distribution is normalized over all DPPC lipids for the entire length of the simulation.

Fig. 11.

Plot of the rotational correlation function of the transition moment vector ($C(t)$) defined as a line connecting atoms C27 to C33 (solid line). The plot is fit with a biexponential decay (eqn (6), dashed line). The parameters of the fit are given in the text.

Fig. 12. Time dependence of mean square displacement of DPPC and DiI. The results are averaged over all the molecules. The MSD of DiI and DPPC are comparable.

Atom charge distribution on the chromophore moiety of DiI-C₁₈(3) obtained from quantum mechanical calculations using Gaussian 03. The total amount of charge on the dye molecule is +1. The alkyl chains of DiI are assigned a charge value of zero similar to the acyl chains of the DPPC molecules. For numbering of atoms refer to Fig. 2. The charge distribution on the head group of DPPC molecules can be obtained from <http://moose.bio.ucalgary.ca/files/dppc.itp>

Table 1

Atom	Charge	Atom	Charge	Atom	Charge	Atom	Charge	Atom	Charge
N19	0.14	C24	0.01	C29	0.30	C34	0.14	C39	0.01
C20	0.10	C25	-0.10	C30	-0.43	C35	-0.03	C40	0.04
C21	-0.04	C26	0.40	C31	0.37	C36	-0.04	C41	-0.06
C22	0.04	C27	-0.06	C32	-0.34	C37	0.03	C42	0.14
C23	0.01	C28	-0.07	C33	0.41	C38	-0.02	N43	0.14