Fluorescent membrane probes' behavior in lipid bilayers: insights from molecular dynamics simulations

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Abstract Fluorescence spectroscopy and microscopy have been used as tools to study membrane biophysics for decades now. Because phospholipids are non-fluorescent, the use of extrinsic membrane probes in this context is commonplace. Two major points of concern arise regarding this matter, namely the incomplete understanding of the probe behavior inside the bilayer and the perturbation of the latter resulting from probe incorporation. To this effect, molecular dynamics (MD) simulations, by providing detailed atomic-scale information, represent a valuable way to characterize the location and dynamics of bilayer-inserted membrane probes, as well as the magnitude of perturbation they induce on the host lipid structure, and several important classes of reporter molecules have been studied in recent years. This article reviews the state of the art of MD simulations of bilayer-inserted fluorescent probes, focusing on the information that has been obtained from previous studies and hinting at future perspectives in this rapidly emerging field.

Keywords Fluorescence · Molecular simulation · Membrane perturbation · Phospholipid

Introduction

The molecular level organization and structure/function relationships in membrane systems pose challenging problems that require a variety of experimental approaches for their study (Gennis 1989). Among these, and due to its sensitivity and versatility, fluorescence came to the fore. The time-scale of fluorescence (sub-nanosecond resolution) is adequate to follow the kinetics of both fast (fluorophore rotation, conformational changes) and relatively slow (translocation, hindered diffusion) processes. Its intrinsic sensitivity is almost unrivalled, and is fully exploited in techniques such as fluorescence correlation spectroscopy and single-fluorophore imaging. Perhaps most importantly, fluorescence spectroscopy is highly versatile, as different approaches (anisotropy, quenching, Förster resonance energy transfer) allow studying such diverse problems of relevance in membrane biophysics as extent of partition to the bilayer, location, rotational and translational dynamics, distribution and aggregation state of membrane components and membrane-active molecules. Fluorescence microscopy adds spatial resolution to the canon, and also, because of the non-destructive nature of fluorescence techniques, is widely used in the study of live cells (Royer and Scarlata 2008; de Almeida et al. 2009).

The basic structural unit of biological membranes is the phospholipid bilayer, and because the vast majority of naturally occurring membrane lipids are non-fluorescent, extrinsic membrane probes are widely used. Design fluorophores often possess convenient photophysical properties, such as absorption and emission in the visible range and high molar absorption coefficient and fluorescence quantum yield, which is especially convenient for microscopy and single-molecule techniques. Some fluorophores are known for their simple decay kinetics, stability, and

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invariance of fluorescence parameters, while others are notable for their sensitivity to the local environment. Taking into account the purpose of a given experiment, one of these types of molecules will be most appropriate (Maier et al. 2002).

However, two major issues arise regarding the use of extrinsic fluorescent probes in membrane studies. First, the behavior of the probe molecules inside the bilayer (what transverse region of the bilayer is the probe sensitive to, its translational and rotational dynamics) is often not fully understood. Second, when interpreting the results of fluorescence experiments, it can be hard to distinguish between legitimate membrane properties and perturbing effects resulting from probe incorporation. Whereas the first point can be addressed experimentally using suitable fluorescence techniques (e.g., differential quenching, dynamical self-quenching, time-resolved anisotropy), the latter issue is best dealt with using a methodology able to simultaneously monitor the probe and lipid molecules independently. Given the sheer amount of work that has been carried out using fluorescent membrane probes, it is surprising that exceedingly few studies have addressed this fundamental matter.

Molecular dynamics (MD) simulations can provide detailed atomic-scale information, and have been extensively used in the study of lipid bilayer structure and dynamics (Ash et al. 2004; Berkowitz 2009). Therefore, they can be used to characterize the location and dynamics of membrane probes, as well as the extent of perturbation they induce on the host lipid structure. Fluorophore properties, such as mass distribution, conformation/orientation, extent of solvation/hydrogen bonding, radial distribution functions, rotational/translational dynamics, and aggregation state, can be monitored. On the other hand, bilayer parameters include area per lipid, density profiles, translational and rotational (headgroup, acyl chain) dynamics, order parameters, orientation of water molecules and lipid headgroups at the interface, and electrostatic potential across the membrane. Comparison between simulation runs in presence and absence of bilayer-incorporated probe allows evaluation of the perturbation induced by the latter. For the purpose of validation of the simulation parameters and protocols, several parameters calculated from simulation can be compared to experimental observables. In addition, unique detailed information can be recovered at the atomic scale. Although the first reported application of atomistic MD simulations of bilayers containing fluorescent probes was reported back in 1997, it was limited to a very short trajectory (<1 ns) (López Cascales et al. 1997). In the last 5 years, studies similar in principle but with much larger scope have focused on several important classes of probes (see Fig. 1

for structures). This work illustrates the wealth of detailed information available from these simulations.

Membrane probes studied using MD simulations

DPH

1,6-diphenylhexatriene (DPH; Fig. 1a) is a rod-shaped molecule whose fluorescence quantum yield and intensity decay show little sensitivity to the lipid phase, unlike the fluorescence anisotropy, which decreases threefold upon melting of the lipid acyl chains. As a consequence, DPH is commonly used as a probe of membrane fluidity (Lentz 1989, 1993). The complex fluorescence anisotropy decays of bilayer-inserted DPH have been analyzed in several systems using sophisticated modeling, and bimodal angular distributions of the long molecular axis, with a maximum parallel and the other perpendicular (in the bilayer mid-plane, between the two monolayer leaflets) to the bilayer normal, have been invoked (Straume and Litman 1987; Mitchell and Litman 1998).

In a pioneering early report (López Cascales et al. 1997), a 72-molecule 1,2-dipalmitoyl-*sn*-3-glycerophosphocholine (DPPC) bilayer was simulated in the presence of either one or three DPH molecules. All atom charges in the DPH molecule were taken as zero. The temperature (350 K) was well above the main transition temperature of this phospholipid ($T_m=314$ K). This study was considerably limited by the short time-scale probed (only ≤ 250 ps were used for analysis). At variance with the bimodal angular distribution model mentioned above, it was observed that the angle between the long molecular axis and the bilayer normal rarely was $>60^\circ$, and no perpendicular orientations were detected. Fast rotation motions (average correlation times of 0.061 ns for end-to-end tumbling and 0.0051 ns for the vector normal to the phenyl rings in the molecule) were obtained. An abnormally high DPH lateral diffusion coefficient ($1.36 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$) was recovered. Significant disordering effects were observed for membranes containing DPH molecules, especially in the region closer to the lipid/water interface. At variance to these, no significant probe effects were observed either in bilayer thickness or the area/lipid molecule.

A more conclusive MD study of DPH in fluid DPPC bilayers (323 K) was reported more recently (Repáková et al. 2004). This time, a 128-lipid bilayer, labeled with 1 or 3 DPH molecules, was simulated for 50 ns. Two approaches for DPH atom charges were used; no charges at all and charges obtained from *ab initio* quantum mechanical calculations, with largely identical results. The extended time scale of this study enabled the authors to observe occasional probe translocation (flip-flop) events. DPH was

Fig. 1 Structures of fluorescent membrane probes mentioned in the text. **a** DPH; **b** Pyrene; **c** PyrPC; **d** C6-NBD-PC; **e** C12-NBD-PC; **f** DiI; **g** BODIPY-cholesterol

found to be located mostly deep in the acyl chain region of the bilayer, with its center of mass average located at $z = 0.75$ nm from the bilayer center, in agreement with quenching studies (Kaiser and London 1998). Broad angular distributions were recovered, and despite a peak being observed for $f(\theta)\sin(\theta)$ (where f is the orientation distribution function) at $\theta \approx 25^\circ$, significant distribution also occurs around $\theta \approx 90^\circ$. However, perpendicular orientations do not correlate with center of mass location near the bilayer center. The correlation function for the long molecular axis has a relaxation time of 4.3 ns, of the order or the rotational correlation times measured by time-resolved fluorescence anisotropy. The calculated lateral diffusion coefficient of DPH ($20 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$) is faster, albeit of the order of that of DPPC ($15 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$), possibly reflecting the increased free volume in the lipid acyl region of the bilayer.

A second part of this study, focusing on the effects of DPH on fluid (323 K) DPPC bilayer properties, was subsequently published (Repáková et al. 2005). MD simulations, again in 128-DPPC bilayers, were now extended to 100 ns, and complemented with experimental differential scanning calorimetry (DSC) and deuterium nuclear magnetic resonance (^2H -NMR) measurements.

DPH effects on the thermotropic phase behavior of DPPC are very minor (very slight decrease of T_m , slight decrease in the corresponding transition enthalpy). ^2H -NMR data suggest that DPH has a very small orientating effect on the DPPC acyl chains. This is confirmed by the MD simulations, which were able to establish that, whereas this effect is indeed modest overall, significant increases in the calculated deuterium order parameter $-S_{\text{CD}}$ (up to 30–50%) were observed for lipid molecules in close proximity (<1.0 nm) of a DPH molecule. Ordering effects become negligible for DPH-DPPC distances >1.5 nm. Thus, by standing mostly upright in the membrane hydrophobic region, DPH produces a local ordering effect on fluid DPPC acyl chains. This is also apparent in the average area/lipid variation as a function of the distance to the nearest probe molecule. A $\sim 2\%$ overall decrease is verified, which is significantly more pronounced for DPPC molecules nearby a probe. Also consistent with this picture is a similar increase in bilayer thickness upon probe incorporation. DPH has negligible influence on interface properties such as membrane electrostatic potential and P-N tilt angle, but affects both lateral diffusion (halving the coefficient value) and rotation of the P-N axis (relaxation time increases by $\sim 10\%$) for nearby DPPC

molecules. Overall, DPH has a small perturbing effect on DPPC fluid bilayers justifying its wide use as a fluorescent membrane probe, though significant local ordering effects are observed.

Pyrene probes

Pyrene (Fig. 1b) is a polycyclic aromatic hydrocarbon notable for its spectroscopic properties, namely the unusually long fluorescence lifetime (>100 ns in a variety of solvents and membrane systems) and ability to form excimers. Due to these distinctive features, both free pyrene and pyrene-labeled lipids have been used in membrane biophysics studies (Somerharju 2002). Three recent reports have used MD simulations to study the behavior of these probes in lipid bilayers. The first (Hoff et al. 2005) combined MD and ^2H -NMR measurements. The simulated system consisted of 128 molecules of 1-palmitoyl,2-oleoyl-*sn*-3-glycerophosphocholine (POPC), with four pyrene molecules inserted inside the membrane and a fifth located in the water region of the box at the start of the MD run, which spanned 25 ns. The latter pyrene molecule was found to insert the POPC bilayer rapidly (<2 ns). Within ~ 8 ns of the start, all five molecules were located slightly inside the headgroup region of the bilayer. This somewhat shallow location agrees with a published experimental fluorescence quenching report (Herrenbauer 2002) and could be due to entropic reasons, as location of this large, rigid molecule in the highly disordered middle region of the membrane would imply considerable acyl-chain ordering and entropy decrease. No flip-flops were observed in the time-scale of the simulation. The molecules were found to have their long axis essentially aligned (within $\pm 30^\circ$) with the bilayer normal. Good agreement between probe order parameter values determined from ^2H -NMR and MD was verified, seemingly indicating that even 25 ns of simulation seem to be enough to cover all types of motions that pyrene performs inside a lipid bilayer.

Two later studies focused both on probe properties and on its effect on the host bilayer. In the first (Repáková et al. 2006), 1, 4, and 6 molecules of the pyrene-tagged lipid 1-palmitoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (PyrPC; Fig. 1c) were simulated in a fluid (323 K) DPPC bilayer (128 total lipid molecules). In this study, no partial charges were used for the pyrene moiety. It was found that, although no significant overall effects were apparent in the average area/lipid molecule, sizeable reductions were detected for lipids located at distances $R < 1.5$ nm to the nearest PyrPC. These effects were as high as $\sim 3\text{--}4\%$ for $R < 1.0$ nm. In accordance, increases in the calculated acyl chain $-S_{\text{CD}}$ were apparent in the same range of R values. Interestingly, the recovered area/PyrPC molecule is even smaller than that obtained for its nearest DPPC neighbors, and the $-S_{\text{CD}}$ value

for the unlabeled *sn*-1 chain is notably high. This indicates that the pyrene moiety increases the order of both neighboring DPPC chains and also the *sn*-1 chain of the lipid molecule to which it is attached. PyrPC incorporation has very minor effects on the DPPC density profiles or bilayer thickness, and the label is mostly found ~ 0.8 nm from the lipid/water interface, in the hydrocarbon region of the bilayer. A broad orientation distribution of the fluorophore is verified, implying a significant fraction of pyrene labels pointed toward the water–lipid interface, causing a kink in the probe *sn*-2 acyl chain. On the other hand, for molecules that do not present this behavior, the pyrene group often interdigitates to the opposite leaflet ($\sim 3\text{--}6\%$ of all configurations). According to the authors, this behavior can cause occasional excimer formation involving PyrPC molecules of different leaflets (as suggested by Fig. 2). Another interesting consequence of pyrene interdigitation is an increase in $-S_{\text{CD}}$ of the nearest DPPC acyl chains ($R < 0.5$ nm) in the other leaflet.

The second study concerned free pyrene, in both fluid (0:128, 1:128, and 3:128 pyrene/lipid ratios, $T=325$ K or 350 K, 20 ns) and gel DPPC (0:120, 1:120, and 3:120 pyrene/lipid ratios, $T=293$ K, 50 ns) bilayers (Čurdová et al. 2007). Partial charges of pyrene atoms were obtained from ab initio quantum mechanical calculations. For all systems, and similar to PyrPC, the overall effects of pyrene on area/lipid and $-S_{\text{CD}}$ are minor. This is because the molar concentrations of pyrene are small. However, significant local (for $R < 1.0$ nm) effects are present. In the fluid phase simulations, acyl chain ordering is verified, similar to that described for PyrPC above. Concerning the gel phase, analysis of $-S_{\text{CD}}$ is rendered difficult because pyrene reduces the tilt angle of nearby DPPC acyl chains, which would per se result in an increased order parameter (as the latter reflects the angle between the chain and the bilayer normal). However, the fraction of trans/gauche defects along the acyl chains of neighboring DPPC molecules

Fig. 2 Snapshot of PyrPC/DPPC bilayers (water molecules omitted) showing “excimer” formation involving PyrPC molecules of different bilayer leaflets. Reprinted with permission from Repáková et al. 2006. Copyright 2006 American Chemical Society

actually increases, meaning pyrene molecules decrease their ordering. Variations of bilayer thickness are consistent with these findings, and pyrene incorporation causes a significant reduction and minor increase in the gel and fluid phases, respectively. Pyrene has broad transverse distribution and orientation profiles, with maxima around acyl chain carbon 5 (distribution) and for $\theta=90^\circ$ (orientation of the pyrene plane relative to the bilayer plane), respectively. Both observations are in agreement with Hoff et al. (2005). In the gel phase, the orientation profile is slightly more complex, with a second maximum being apparent at 50° (probably reflecting the tilt of the DPPC acyl chains). No significant changes in lateral diffusion coefficient of DPPC were observed upon pyrene insertion. No clustering of pyrene molecules was observed, possibly as a result of time scale limitations.

NBD probes

Whereas the previous examples refer to apolar, aromatic fluorophores, the use of fluorescent membrane probes bearing polar design fluorophores has become very common, especially since the 1980s. Among them, a popular family is that of phospholipids labeled with the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) fluorophore in one of the acyl chains. NBD derivatives are commercially available for all major phospholipid classes, and have been used extensively as fluorescent analogues of native lipids in biological and model membranes to study a variety of processes (Chattopadhyay 1990; Mukherjee et al. 2004).

The behavior of two of these probes, the acyl chain-labeled phosphatidylcholine derivatives 1-palmitoyl,2-[12-amino]hexanoyl-*sn*-glycero-3-phosphocholine (C6-NBD-PC; Fig. 1e) and 1-palmitoyl,2-[12-amino]dodecanoyl-*sn*-glycero-3-phosphocholine (C12-NBD-PC; Fig. 1f) was simulated in fluid DPPC bilayers (0:64, 1:63, 4:60 NBD-PC/DPPC bilayers, 323 K, 100 ns). The study was split in two parts, the first one mainly concerned about location and dynamics of the probes (Loura and Prates Ramalho 2007) and the second focused on their effect on the host bilayer properties (Loura et al. 2008). Parametrization of the NBD moiety required energy minimization for geometry and *ab initio* quantum mechanical calculations for partial atomic charges. Simulations were complemented with time-resolved fluorescence anisotropy (Loura and Prates Ramalho 2007) and DSC (Loura et al. 2008) measurements, to obtain experimental information on probe rotational dynamics and perturbation of the thermotropic behavior of DPPC, respectively. The most important findings can be summarized as follows:

1. NBD loops in the direction of the interface. It has a broad transverse distribution in the bilayer, with a

maximum around the glycerol backbone/carbonyl region (in accordance with fluorescence quenching and ^2H -NMR results; Chattopadhyay and London 1987; Huster et al. 2001), the nitro group being closest to the interface (see Fig. 3);

2. the NBD NH group is involved in H-bonding to PC glycerol backbone O atoms;
3. the theoretical rotational dynamics of NBD agrees with experimental fluorescence anisotropy decays;
4. lateral translation diffusion of NBD-PC is identical to that of DPPC;
5. whereas important effects (see Table 1) are observed for high (~ 6 mol%) probe content, milder perturbation of fluid PC structure and dynamics is expected for ~ 1 mol% or less probe, the concentrations most often used experimentally. Regarding the range of the induced perturbations, as judged by $-S_{\text{CD}}$ values, they occur mostly in molecules in close contact with probes. This is especially true for C6-NBD-PC. Because the labeled *sn*-2 chain of this probe does not extend nearly so much

Fig. 3 Typical snapshots of C6-NBD-PC (a) and C12-NBD-PC (b) containing bilayers. Water molecules are omitted. Reprinted with permission from Loura and Prates Ramalho 2007. Copyright 2007 Elsevier

Table 1 Summary of probe effects on bilayer properties, as obtained from MD simulations

Probe	area/ lipid	Bilayer thickness	$-S_{CD}$	[Electrostatic potential]	Angle between P-N vector and bilayer normal	DPPC lateral diffusion coefficient	DPPC rotational dynamics
DPH ^a	↓	↑	↑	0	0	↓	↓
PyrPC ^b	↓	↑	↑	—	—	—	—
Pyrene ^c	↓	↑	↑	—	—	0	—
NBD-PC ^d	↑	0	↓	↑	0	↓	↓ ^e
DiI ^f	0	↑	↑	↑	↓	—	—
BODIPY-cholesterol ^g	0	0	↓	—	—	—	—

↑ Increase, ↓ decrease, 0 non-significant effect, — not reported

^a Repáková et al. 2005

^b Repáková et al. 2006

^c Čurdová et al. 2007

^d Loura et al. 2008

^e For C12 derivative (no significant effect for C6 probe)

^f Gullapalli et al. 2008

^g Hölttä-Vuori et al. 2008; relative to a DPPC bilayer with identical cholesterol content

across the bilayer as that of C12-NBD-PC (see Fig. 3), the disordering effect of the former is mostly concentrated in its immediate vicinity, whereas the large extension of the C12-NBD-PC *sn*-2 chain allows for a more uniform distribution of the perturbation among additional layers of neighbors;

- however, even for these smaller amounts, large alterations (transition temperature shifts, loss of cooperativity), are evident in the thermotropic behavior of NBD-PC labeled PC bilayers, as measured by differential DSC.

DiI

1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine (DiIC₁₈(3) or DiI) is the most representative member of the carbocyanine family of dyes. These molecules have very high molar absorption coefficients ($\sim 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) and are used as membrane potential probes and as stains in cell studies. They have also been extensively used as probes of bilayer dynamics and structure (Wolf 1988). The behavior of DiI in fluid DPPC bilayers was recently studied by MD (Gullapalli et al. 2008). The simulated systems were 0:128, 2:126, and 4:124 DiI/DPPC bilayers, at 323 K, for 40 ns in each case. Partial atom charges on the DiI headgroup (global charge +1) were obtained from ab initio quantum mechanical calculations. A fourth simulation, similar to the 4:124 case, but in the absence of any partial charges on the DiI head group, was also performed for the sake of comparison.

It was found that, whereas DiI does not affect the area/lipid molecule significantly, membrane thickness was found

to increase by 3–5% (~ 0.1 – 0.2 nm) in presence of incorporated probe. In accordance, DiI was found to increase $-S_{CD}$ for both *sn*-1 and *sn*-2 DPPC acyl chains. Similar to the NBD fluorophore, it was found that the center of mass of the DiI headgroup was distributed between 0.3 and 2 nm from the center of the bilayer, with a maximum at 1.26 nm, in the glycerol/upper acyl chain region, and below the phospholipid headgroup region. As pointed out by the authors, this result is in contrast to some popular cartoon representations of DiI but consistent with DiI's increase in fluorescence quantum yield when incorporated into lipid bilayers. Flip-flop was observed in the control simulation with uncharged DiI atoms but not in the other simulation runs, indicating that the overall positive charge prevents the occurrence of probe translocation. The angle between the chromophore long axis (uniting the two C6 rings) and the bilayer normal had a broad average distribution, with a maximum at 77° , almost parallel to the bilayer plane, in accordance with fluorescence polarization data (Axelrod 1979). Addition of DiI leads to ordering of water at the interfacial region (mainly as a consequence of increase in the thickness of the bilayer), decrease in the angle between the P-N vector and the bilayer normal, and significant variation of electrostatic potential across the membrane from -0.56 V (DPPC) to -0.69 V (4 DiI:124 DPPC). The latter values can be compared with the above described NBD study (Loura et al. 2008) for which a smaller variation was observed for higher labeling ratios (-0.55 V for DPPC, -0.58 V for 4 C6-NBD-PC:60 DPPC, -0.64 V for 4 C12-NBD-PC:60 DPPC). The decay of the autocorrelation function of the chromophore axis agrees with literature anisotropy decay parameters of related

carbocyanines, and DiI has a lateral diffusion coefficient identical to that of DPPC.

BODIPY-PC

Cholesterol is a major component of mammalian cell membranes, and its action upon the physical properties of lipid bilayers has been studied actively in the last four decades. More recently, its implication in raft domains (Simons and Ikonen 1997) has further increased this interest. Several fluorescent analogs of cholesterol are commercially available, but some of them behave very differently from cholesterol in lipid bilayers, preferring the cholesterol-poor liquid disordered phase to the cholesterol rich liquid ordered phase (e.g., Loura et al. 2001). Recently, a cholesterol derivative bearing the 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) fluorophore (BODIPY-cholesterol; see Fig. 1g) was found to partition into ordered domains in model membranes (Shaw et al. 2006).

A recent work using BODIPY-cholesterol combined live cell imaging (which established that the probe closely mimics the membrane partitioning and trafficking of cholesterol) with MD simulations (Hölttä-Vuori et al. 2008). Focusing on the latter, systems with both DPPC and *N*-palmitoylsphingomyelin (SM) were simulated at 323 K, for ≥ 60 ns in each case. In a first set of simulations (3.125% sterol), bilayers of 124 DPPC or SM and 4 sterol molecules were simulated. The used sterol was either cholesterol or BODIPY-cholesterol. In a second set of simulations (20% total sterol), bilayers of 128 SM or DPPC and 32 cholesterol molecules were simulated, as well as bilayers with 128 SM or DPPC, 28 cholesterol and 4 BODIPY-cholesterol molecules. The BODIPY moiety contains a boron atom, not originally included in the used force field, which had to be adapted to this effect. Partial atom charges in the fluorophore were obtained from ab initio quantum mechanical calculations.

It was found that the effect of BODIPY-cholesterol on bilayer properties matched that of cholesterol. Differences in the effect of sterol on area/lipid and membrane thickness were not significant. Differences in the effect of sterol on $-S_{CD}$, while noticeable, were relatively minor (BODIPY-cholesterol was slightly less efficient in ordering fluid lipid acyl chains than cholesterol; the perturbation range of both molecules is in the order of ~ 1.0 nm). The distribution of the angle between the vector uniting the ends of the sterol ring system and the bilayer normal was predominantly at $< 45^\circ$ in all cases. Distributions of BODIPY-cholesterol were slightly wider and displaced to larger angles as compared to those of cholesterol. On the other hand, the angle between the longer axis of the BODIPY moiety itself and the bilayer is predominantly at $> 45^\circ$. Given that the maximum of the mass distribution profile of the BODIPY

moiety is in the middle of the bilayer, one can expect to find the probe molecules with their chromophore located deep along the bilayer midplane. This is the predominant configuration, corresponding to an upright orientation of the steroid ring system (similar to cholesterol); for low sterol bilayers, a significant probe population displays a more tilted steroid ring system, together with a slight upwards loop of the BODIPY moiety. Remarkably, in more ordered bilayers (containing SM instead of DPPC, or 20 mol% sterol instead of 3.125%), this second population tends to vanish, giving rise to unimodal orientation distributions of BODIPY-cholesterol, as the lipid acyl chains become more ordered. This means that BODIPY-cholesterol is an especially good mimic of cholesterol for more ordered environments, which is an important asset for a raft reporter. The location and orientation of cholesterol itself is not affected by the presence of a smaller amount of BODIPY-cholesterol.

Concluding remarks

Table 1 summarizes and compares the effects on fluid DPPC bilayer properties of the probes simulated in the reports mentioned. Note that many of these effects are minor and most noticeable for DPPC molecules close to a probe (e.g., DPH, pyrene, PyrPC) or for high probe concentration (such as area/lipid in NBD-PC).

Although MD simulations of lipid bilayers containing fluorescent probes are still few in number, virtually all studies are very recent (last 5 years), indicating that this is fast becoming a very active field within theoretical modeling of membranes. The power of this approach is clearly visible from the described works. A number of experimental observables could be verified, therefore validating the methodology. Most importantly, unique information has been retrieved, both on probe properties and regarding their effect on the host bilayer. The combination of simulation of the probes' behavior and experiments designed taking this behavior into consideration has potential to provide accurate insights on membrane structure and function at a molecular level.

Whereas the first published reports concerned highly apolar fluorophores, simpler in structure and easier to model, attention is gradually turning to polar, complex design fluorophores. Most simulations are still being carried out in liquid disordered bilayers. However, given the recognized importance of ordered and even rigid domains such as lipid rafts (Simons and Ikonen 1997) and ceramide platforms (Zhang et al. 2009), where the problem of finding adequate fluorescent reporters is more critical, it is expected that simulations in liquid ordered and gel phases will become more frequent in the near future.

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References

- Ash WL, Zlomislic MR, Oloo EO, Tieleman DP (2004) Computer simulations of membrane proteins. *Biochim Biophys Acta* 1666:158–189
- Axelrod D (1979) Carbocyanine dye orientation in red cell membrane studied by microscopic fluorescence polarization. *Biophys J* 26:557–573
- Berkowitz ML (2009) Detailed molecular dynamics simulations of model biological membranes containing cholesterol. *Biochim Biophys Acta* 1788:86–96
- Chattopadhyay A (1990) Chemistry and biology of N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-labeled lipids: fluorescent probes of biological and model membranes. *Chem Phys Lipids* 53:1–15
- Chattopadhyay A, London E (1987) Parallax method for direct measurement of membrane penetration depth utilizing fluorescence quenching by spin-labeled phospholipids. *Biochemistry* 26:39–45
- Čurdová J, Čapková P, Plášek J, Repáková J, Vattulainen I (2007) Free pyrene probes in gel and fluid membranes: perspective through atomistic simulations. *J Phys Chem B* 111:3640–3650
- de Almeida RF, Loura LMS, Prieto M (2009) Membrane lipid domains and rafts: current applications of fluorescence lifetime spectroscopy and imaging. *Chem Phys Lipids* 157:61–77
- Gennis RB (1989) *Biomembranes: molecular structure and function*. Springer, New York
- Gullapalli RR, Demirel MC, Butler PJ (2008) Molecular dynamics simulations of DiI-C₁₈(3) in a DPPC lipid bilayer. *Phys Chem Chem Phys* 10:3548–3560
- Herrenbauer M (2002) Biosorption of Polycyclic Aromatic Hydrocarbons (PAH) to microorganisms and liposomes. Shaker, Aachen
- Hoff B, Strandberg E, Ulrich AS, Tieleman DP, Posten C (2005) ²H-NMR study and molecular dynamics simulation of the location, alignment, and mobility of pyrene in POPC bilayers. *Biophys J* 88:1818–1827
- Hölttä-Vuori M, Uronen RL, Repáková J, Salonen E, Vattulainen I, Panula P, Li Z, Bittman R, Ikonen E (2008) BODIPY-cholesterol: a new tool to visualize sterol trafficking in living cells and organisms. *Traffic* 9:1839–1849
- Huster D, Müller P, Arnold K, Herrmann A (2001) Dynamics of membrane penetration of the fluorescent 7-nitrobenz-2-oxa-1, 3-diazol-4-yl (NBD) group attached to an acyl chain of phosphatidylcholine. *Biophys J* 80:822–831
- Kaiser RD, London E (1998) Location of diphenylhexatriene (DPH) and its derivatives within membranes: comparison of different fluorescence quenching analyses of membrane depth. *Biochemistry* 37:8180–8190
- Lentz BR (1989) Membrane “fluidity” as detected by diphenylhexatriene probes. *Chem Phys Lipids* 50:171–190
- Lentz BR (1993) Use of fluorescence probes to monitor molecular order and motions within liposome bilayers. *Chem Phys Lipids* 64:99–116
- López Cascales JJ, Huertas ML, García de la Torre J (1997) Molecular dynamics simulation of a dye molecule in the interior of a bilayer: 1, 6-diphenyl-1, 3, 5-hexatriene in dipalmitoylphosphatidylcholine. *Biophys Chem* 69:1–8
- Loura LMS, Prates Ramalho JP (2007) Location and dynamics of acyl chain NBD-labeled phosphatidylcholine (NBD-PC) in DPPC bilayers. A molecular dynamics and time-resolved fluorescence anisotropy study. *Biochim Biophys Acta* 1768:467–478
- Loura LMS, Fedorov A, Prieto M (2001) Exclusion of a cholesterol analog from the cholesterol-rich phase in model membranes. *Biochim Biophys Acta* 1511:236–243
- Loura LMS, Fernandes F, Fernandes AC, Prates Ramalho JP (2008) Effects of fluorescent probe NBD-PC on the structure, dynamics and phase transition of DPPC. A molecular dynamics and differential scanning calorimetry study. *Biochim Biophys Acta* 1778:491–501
- Maier O, Oberle V, Hoekstra D (2002) Fluorescent lipid probes: some properties and applications (a review). *Chem Phys Lipids* 116:3–18
- Mitchell DC, Litman BJ (1998) Molecular order and dynamics in bilayers consisting of highly polyunsaturated phospholipids. *Biophys J* 74:879–891
- Mukherjee S, Raghuraman H, Dasgupta S, Chattopadhyay A (2004) Organization and dynamics of N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-labeled lipids: a fluorescence approach. *Chem Phys Lipids* 127:91–101
- Repáková J, Čapková P, Holopainen JM, Vattulainen I (2004) Distribution, orientation, and dynamics of DPH probes in DPPC bilayer. *J Phys Chem B* 108:13438–13448
- Repáková J, Holopainen JM, Morrow MR, McDonald MC, Čapková P, Vattulainen I (2005) Influence of DPH on the structure and dynamics of a DPPC bilayer. *Biophys J* 88:3398–3410
- Repáková J, Holopainen JM, Karttunen M, Vattulainen I (2006) Influence of pyrene-labeling on fluid lipid membranes. *J Phys Chem B* 110:15403–15410
- Royer CA, Scarlata SF (2008) Fluorescence approaches to quantifying biomolecular interactions. *Methods Enzymol* 450:79–106
- Shaw JE, Epand RF, Epand RM, Li Z, Bittman R, Yip CM (2006) Correlated fluorescence-atomic force microscopy of membrane domains: structure of fluorescence probes determines lipid localization. *Biophys J* 90:2170–2178
- Simons K, Ikonen E (1997) Functional rafts in cell membranes. *Nature* 387:569–572
- Somerharju P (2002) Pyrene-labeled lipids as tools in membrane biophysics and cell biology. *Chem Phys Lipids* 116:57–74
- Straume M, Litman BJ (1987) Equilibrium and dynamic structure of large, unilamellar, unsaturated acyl chain phosphatidylcholine vesicles. Higher order analysis of 1, 6-diphenyl-1, 3, 5-hexatriene and 1-[4-(trimethylammonio)phenyl]- 6-phenyl-1, 3, 5-hexatriene anisotropy decay. *Biochemistry* 26:5113–5120 Erratum in: *Biochemistry* 26:8030
- Wolf DE (1988) Probing the lateral organization and dynamics of membranes. In: Loew LM (ed) *Spectroscopic membrane probes*, vol I. CRC, Boca Raton, pp 193–220
- Zhang Y, Li X, Becker KA, Gulbins E (2009) Ceramide-enriched membrane domains - structure and function. *Biochim Biophys Acta* 1788:178–183