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Molecular modeling of lipid probes and their influence on the membrane

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Abstract

In this review a number of Molecular Dynamics simulation studies are discussed which focus on the understanding of the behavior of lipid probes in biomembranes. Experiments often use specialized probe moieties or molecules to report on the behavior of a membrane and try to gain information on the membrane as a whole from the probe lipids as these probes are the only things an experiment sees. Probes can be used to make NMR, EPR and fluorescence accessible to the membrane and use fluorescent or spin–active moieties for this purpose. Clearly membranes with and without probes are not identical which makes it worthwhile to elucidate the differences between them with detailed atomistic simulations. In almost all cases these differences are confined to the local neighborhood of the probe molecules which are sparsely used and generally present as single molecules. In general, the behavior of the bulk membrane lipids can be qualitatively understood from the probes but in most cases their properties cannot be directly quantitatively deduced from the probe behavior.

Keywords: lipids, simulations, fluorescence, EPR, NMR, MD

1. Introduction

A wide variety of experiments in biomembranes needs specially designed lipids with probes chemically attached to perform the experiment and only the behavior of the probe molecules is seen in the experiments [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13]. Regularly it is assumed that the probe molecules behave essentially the same way as the lipids and membranes they are probing. But it is easy to see that this cannot exactly be the case. Assume for example a diffusion experiment where the probe molecule (often a fluorescent probe) has a much larger molecular weight than the surrounding lipids as the lipid has to carry the fluorescent marker with it. Phase information or information on the type of diffusion can normally be reliably obtained from such an experiment.

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However, the actual quantitative diffusion coefficient of the probe molecule will be different from the surrounding lipids even in the case of ideal mixing. The phase of the lipids can often be deduced as at the phase transition the diffusion drops by several orders of magnitude such that small quantitative differences are not crucial. But for a correct calibration of the diffusion dynamics or other properties we need to understand how probes are behaving differently from lipids.

Molecular modeling can help bridge the gap between the experimental data gained on the probe and obtaining information on the bulk lipids if experiments and simulations are performed in tandem. Molecular Dynamics (MD) simulations are regularly used to capture the behavior of biological macromolecules in full atomic detail, but their computational demands, combined with the challenge of appropriately modeling the relevant physics, sometimes restrict their reliability and accuracy. Dramatic recent improvements in speed and the development of better models have enabled atomic level simulations on timescales up to milliseconds that capture key biochemical and biophysical processes. MD can serve as a computational microscope, revealing biomolecular mechanisms at spatial and temporal scales that are difficult to observe experimentally [14] such that it is an ideal counterpart to real microscopes. MD is well–established for biomolecular studies. It has been used widely to study the behavior of lipids and their interactions [15, 16, 17, 18, 19, 20, 21, 22]. Most simulations on biomembranes and lipid assemblies are performed in atomistic detail where every (at least non-hydrogen) atom is represented, this is the level of detail needed for studying probe lipids as we largely expect local deviations from bulk behavior.

This review aims to summarize simulation efforts on lipid probes over the last decade in order to inform the reader on the current state of the art. Another review of a part of this field has been published a few years ago [23] which, however, focussed on fluorescent probes exclusively. Here we focus particularly on the effects of the probes on the other lipids and differences in behavior between probe lipids and bulk lipids and we discuss also EPR active probe molecules. This review is focused on classical molecular dynamics. There are many other techniques including electron structure calculations or Monte Carlo which are not discussed here.

There are a few experimental studies which investigated the influence of lipid probes on the system. In a study of fluorescence quenching lipid–probe interactions between the non–fluorescent substrate and the lipid, which affect the observed rate of change of fluorescence after addition of lipids to DHR (dihydrorhodamine 123) and DCFH (Dihydrodichlorofluorescein) (for definitions see figure 1) was found [24]. These interactions depend on a large variety of parameters including sample collection and storage, types and concentrations of lipid and fluorescent probe, as well as pH. One assay yielded reproducible measurements despite fluorescence quenching, while the other had rather large experimental variability. Furthermore, the lipid–probe interactions varied according to the introduced level of inflammation. In another study using deuterium nuclear magnetic resonance spectroscopy (^{2}H NMR), it was found that trace amounts of the carbocyanine probe DiIC_{12} which is used as NMR marker were

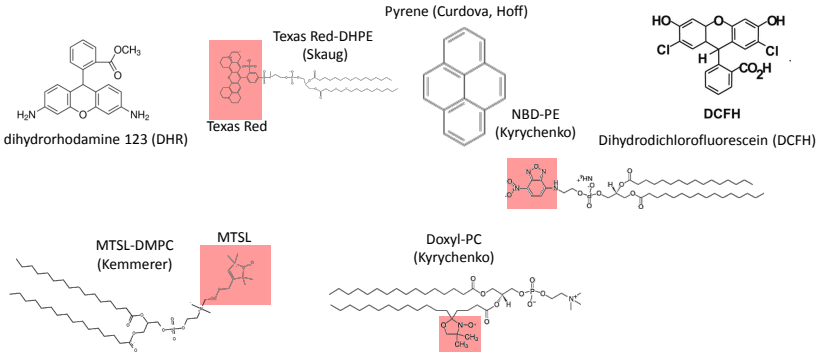

Figure 1: Some of the molecules and moieties used as probes in membranes and discussed in this review. The fluorescent or EPR active moieties connected to other molecules are marked with a pink background. Top row: Fluorescent probes including Dihydrorhodamine 123, Texas Red DHPE, NBD-PE, Pyrene and Dihydrodichlorofluorescein, Bottom Row: EPR Spin probes. Pyrene has also been studied in NMR experiments. The names in parentheses are the researchers who studied the corresponding systems computationally.

enough to alter the phase coexistence of a 30:30:35 DPPC:DOPC:cholesterol membrane, while other probes like Laurdan, Naphthopyrene, and another carbocyanine probe $DiOC_{18}$, did not affect the membrane appreciably [25]. These experimental results make it clear that there is no generic behavior of probe lipids in a membrane and detailed MD studies can be very useful to interpret experiments.

Fluorescent or EPR–spinactive molecules or moieties are very common tools to study the behavior of lipids in membranes. They can be attached to lipids or proteins or added to the membrane as a separate component and can then be monitored with a variety of fluorescence microscopy and optical spectroscopy techniques [26, 27, 28, 29], with EPR [30, 31, 32, 33, 34, 35, 36], and NMR techniques [37, 38]. Figure 1 shows examples of such probe moieties which are abundant in experiments and some of which have been studied computationally. Typically, the focus of a fluorescence experiment is not the fluorophore itself, but the other molecules that make up the lipid membrane. The probes can be thought of as an impurity, a "necessary evil" that allows the measurement of static and dynamic membrane properties of interest. This of course only makes sense if we can assume that the probe does not alter the behavior of the membrane molecules in a dramatic way.

Molecular dynamics simulation is perfectly suited to address the questions associated of if probe molecules correctly represent the "average" behavior of a lipid membrane or if the probe alters its environment or even changes the phase behavior of the lipids around it. Molecular dynamics can provide atomistic detail over length scales of individual to hundreds or thousands of lipids and time

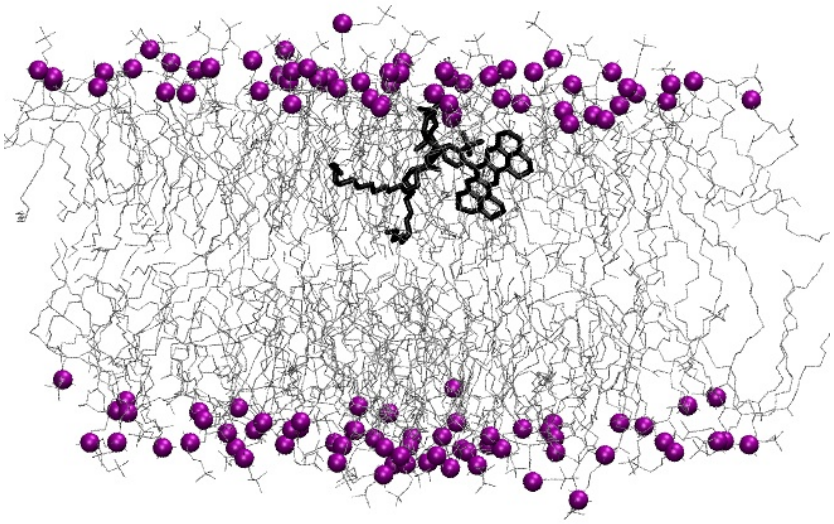

Figure 2: Typical computational system of a biomembrane containing a single probe molecule (here Texas Red DHPE). Reprinted with permission from [42]. Copyright 2009 American Chemical Society.

scales from picoseconds to hundreds of nanoseconds. We expect that the largest influence of the probe molecules is in the local neighborhood of a given lipid, thus we need full spatial resolution which leads to atomistic simulations. Knowing how a fluorescent molecule interacts with the molecules of actual interest – the lipids around it – is particularly critical in single molecule studies [39] as one tries to deduce typical behavior from the observation of one individual molecule which is even of another chemical species. In single molecule tracking experiments [6, 40] a single fluorescent molecule is imaged in time sequence to determine its rotational and translational diffusion behavior. The advantage of such single molecule studies over ensemble techniques is their ability to reveal the statistical distribution of behavior that is averaged over in ensemble measurements [41]. But to interpret such experiments reliably it is important to know that subtle features in the data are caused by the membrane characteristics and not by the fluorophore itself.

Figure 2 shows a visualization of a typical simulation system where one probe lipid is embedded in a bilayer of "regular" lipids. The thick black lines represent one Texas Red marked DHPE lipid. Texas Red is a very abundantly used fluorophore. The purple balls mark the phosphate groups of the non–fluorescent lipids to identify the interface between the aqueous region and the hydrophobic region. It is clear that the chemistry of the fluorophore which often bases on fused aromatic rings is different from any other chemistry in the system. It is known that aromatics prefer the interfaces between water and oil (alkanes) [43]. So we would expect as is seen here that the aromatic

dye segments locate at the interface and may disrupt the lipid packing in their vicinity.

2. Fluorescent Probes

Fluorescent probes which enable fluorescent microscopy techniques are arguably the most abundant type of probe lipids used experimentally so we will first discuss this molecular class. Fluorescent probes can, e.g., be used to study the phase behavior of lipid mixtures because many probe molecules partition preferentially to one of the lipid phases which allows visualization of the phase separation as now the phase with the higher abundance of fluorophores is lighter and the other phase is darker [3, 8, 9, 29]. However, it has experimentally been reported that attachment of a fluorescent label to higher order lipids and eliminate a molecule's ability to partition with other higher order lipids [44] and therefore it might be that one gets un–intuitive phase identification. In order to avoid this, simulations of the probes with different neighborhoods are needed to determine which lipids (or mixtures) are preferred by a given probe.

Many fluorescent molecules, particularly the ones which are used in single molecule studies, are of the xanthene family of dyes [45]. These are polycyclic aromatics and can be attached chemically to a lipid headgroup. One expects that this headgroup then either sticks out into the water phase as it is polar or even charged. In many cases (see Fig. 2) the aromatics actually locate at the interface even if they are charged. The probably most famous example of the xanthenes is Texas Red (sulforhodamine 101 acid chloride) which is available commercially already attached to a lipid in the form of Texas Red–DHPE [46]. Texas Red–DHPE is highly sought after for its high quantum yield and stable fluorescence and therefore has been used in numerous lipid bilayer studies [47, 48, 49, 50]. Texas Red leads to a headgroup labeled lipid which carries a single negative charge as the choline group of typical phosphocholine lipids is missing and replaced by the dye. The dye part has a molecular weight of 652.2 g/mol, which is very close to the molecular weight of DHPE, 691.97 g/mol. Thus, the combined molecule is about twice as heavy as the neighbors in the membrane.

Although they are usually incorporated at low concentration, several experimental studies suggest that fluorescent labels could alter the membrane behavior [24, 25]. This was investigated for the example of Texas Red also computationally [42, 51]. First a model of TR–DHPE had to be developed. This is the standard case as such models are rarely directly available in the literature. Particularly the charge distribution on the dye needs to be paid attention to. TR-DHPE is composed of two basic units like most labeled lipids, the Texas Red fluorescent moiety and the DHPE moiety (Fig. 1). The simulation parameters for the lipid part can be taken directly from the large literature on biomembrane modeling. The Berger model [52] is often a good choice for the lipid part and was used in this case. In this case the interaction parameters of this model were also used for the atom types, force constants, dihedral values, and nonbonded parameters of the Texas Red dye atoms for consistency. However, the charge distribution did not exist before and density functional

calculations (DFT) were needed. The complete headgroup including the dye and the phosphate was modeled using DFT and partial charges were assigned using the Merz–Kollman–Singh method [53] which fits the quantum mechanically obtained electrostatic potential to a point charge model which then can be used in regular atomistic MD. Another group used a very similar approach for the development of a BODIPY–PC model [54] except that they used CHELPG charges [55] and the CHARMM force–field [56].

The final model was then used to simulate TR–DHPE in 1,2–dipalmitoyl–sn–glycero–3–phosphocholine (DPPC) bilayers with 127 or 511 DPPC lipids, i.e. only one lipid with a fluorophore was initially used [42]. Texas Red was found to reside in the upper acyl chain region of the bilayer. Based on its position in the bilayer, Texas Red decreased the order in the upper part of the acyl chain in the neighboring lipids. This disturbance was very localized and its spatial extension was determined in detail based on the average area per lipid at different radial distances from the TR–DHPE molecule. Lipids in the same leaflet as the TR–DHPE were categorized according to their distance from TR–DHPE molecule at each time frame. The bilayer surface was divided into concentric rings around the TR–DHPE. It turns out that in the local neighborhood the area is greatly increased to about 0.72 nm^2 but quickly decreases radially outward to an unperturbed system at 0.66 nm^2 in the 1:127 system. However, there were significant finite size effects in the 1:127 system such that results in such system sizes are questionable. One complication was that the standard assumption that Texas Red spans the entire leaflet in which it resides homogeneously is not always valid.

It was also found that the Texas–Red lipids bind to one or more DPPC lipid [42]. At higher concentrations (5 mol %) TR–DHPE and DPPC binding is due to electrostatic interactions [51]. On average, TR–DHPE is bound to 1.2 DPPC molecules. Binding reduces the diffusion coefficient of TR–DHPE by about one third relative to unlabeled DPPC molecules, thus quantitative estimates from e.g. single molecule tracking experiments have to take this into account. The binding occurs between the phosphate group of an unlabeled phospholipid and the aryl group of the Texas Red and leads to a mini cluster of a labeled lipid and $1 - 3$ unlabeled lipids. An average residence time of a few tens of ns was found but in some instances the binding lasted over more than 0.5 μs. There are two different unique binding locations for DPPC on the Texas Red. As seen in Fig. 2 TR-DHPE adopts a bent configuration in the bilayer, with the lipid tails and the xanthene structure in the upper hydrophobic core of the bilayer. This allows the more highly charged, phosphate and sulfur groups to remain at the interphase. The closest (bound) DPPC locates in the bend of TR–DHPE, between the lipid and xanthene sections of TR-DHPE. The binding locations can be identified by radial distribution functions and from this one can determine a potential of mean force to determine binding strengths (see Fig. 3). The binding is slightly stronger than thermal energy at relevant temperatures (room to body temperature) whereas the interactions between normal lipids do not show this effect.

This clearly indicates that we have to be very careful with adding different

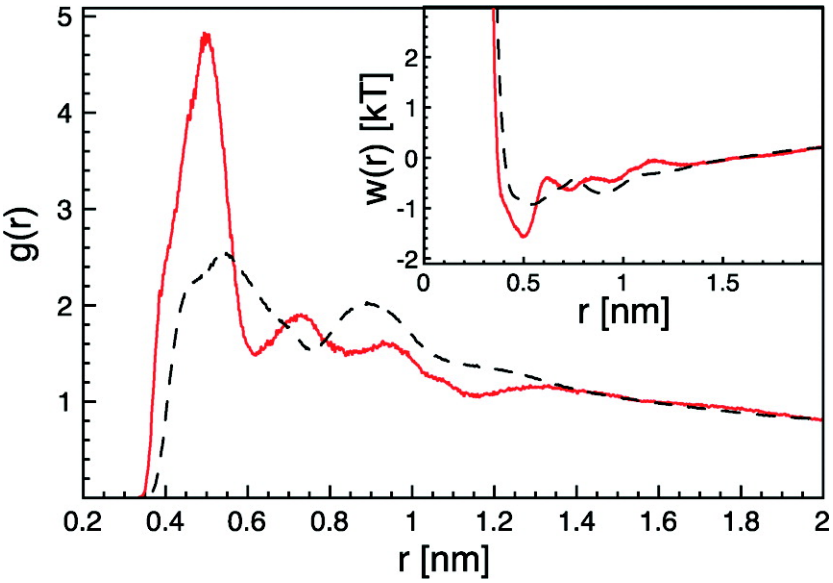

Figure 3: Radial distribution function between TR-DHPE DPPC (solid red). For comparison, the dashed black line is the radial distribution function between DPPC. Reprinted with permission from [51]. Copyright 2011 American Chemical Society.

molecules – such as probes – into a bilayer. They can not only locally disturb the bilayer structurally but they can actually change the local thermodynamics as they might bind other molecules as additional interactions are added.

This study enabled not only an analysis of local perturbations but made it clear that there are actual phase behavior effects of such binding to probe molecules. Putzel et al [57] developed a thermodynamic theory based on a Flory Huggins model [58]. The theory uses clusters of bound molecules to determine shifts in the phase boundaries of lipids. Using the average number of 1.2 lipids bound to a Texas Red leads to a change in the phase behavior and a shift of the phase temperatures can be calculated. Qualitatively, the addition of small amounts (like 1–5 mol%) of TR–DHPE increases the critical temperature. Assuming a DPPC/unsaturated lipid and cholesterol mixture and TR–DHPE preferably binding to the saturated lipid, the critical temperature would increase, but the critical cholesterol concentration would decrease. The temperature scale can be calibrated based on a phase diagram from NMR experiments [59] and one finds that the addition of 1 mol% TR–DHPE increases the critical temperature on the order of 5 K [51].

Texas–Red is clearly not the only or first probe which has been studied in membranes. Curdova et al, e.g., studied free – not chemically attached to lipids – pyrene probes in both gel and fluid phospholipid membranes [60]. For a free probe one first has to validate that the probe actually enters the membrane and it turned out that free pyrene molecules prefer the upper hydrophobic acyl chain

region close to the glycerol groups. The orientation of the pyrene depends on the phase of the membrane. In the fluid (L_α) phase, pyrenes orient along the membrane normal, in the gel phase, the orientation is following the non–zero lipid tilt. Also pyrenes are shown to locally perturb the membrane structure. In the gel phase, pyrenes break the local packing of lipids and decrease the ordering of lipid acyl chains around them, while, in the fluid phase, pyrenes increase the ordering of nearby acyl chains, thus having an opposite effect. This is actually very similar to the behavior of sterols in the membrane where also the rigid but asymmetric structure orders disorder lipids and disorders ordered lipids [60, 61].

Experiments have shown that 2–(2–pyridyl)–5–methylindole (5M–PyIn–0) and 2–[2–(4,6–dimethylpyrimidyl)]–indole (DMPmIn–0) show enhanced fluorescence when they partition into the membrane in contrast to the aqueous phase [62]. These are not chemically bound to lipids and can move between the water and the hydrophobic interior. MD simulations showed that this can be explained by a decrease in the number of hydrogen bonds between the excited fluorophore and water. This causes then quenching of the fluorescence. The simulations showed that both molecules bind quickly to a membrane and partition deeply into the bilayer such that they are shielded from water interactions. The simulations determined the free energy profile of the fluorescence markers using umbrella sampling which revealed a region of low free energy about 1–1.5 nm from the bilayer center [62]. This application shows another advantage of simulations as otherwise a direct measurement of a free energy as a function of position is essentially impossible without adding significant artificial interactions. Another example of fluorescence depending on the neighborhood of the fluorophore is is 2,6–bis(1H–benzimidazol–2–yl)pyridine (BBP) which is almost non–fluorescent in water but strongly fluorescent in hydrophobic environments due to different hydrogen bonding patterns. To understand the binding interactions of such a probe with a membrane on the molecular level computer simulations used two different simulation approaches. Standard MD was first used to study how diffusion drives the probe into the membrane and then the binding free energy was measured by umbrella sampling where then both approaches agreed in their fundamental results that the preferred location of BBP in the interface just behind the lipid headgroups on the initial hydrophobic part. MD simulations also revealed a restricted permeability of water molecules into this region of a POPC bilayer [63].

One particular fluorescence technique is Fluorescence Interference Contrast (FLIC) microscopy which can determine the orientation of the reporter molecules [64, 65, 10]. Using a combination of MD and FLIC it was shown that the orientation of long glycopolymers (30 nm) which are used as FLIC reporters depends on the properties of the probe moieties attached. Such a probe could be Alexa Fluor 488 which projects on average away from the lipid bilayer in the water phase. This can be explained by random entropy dominated orientations which due to the geometrical bias of the membrane leads to an average normal orientation. Reporter molecules which were terminated with Texas Red lie flat at the membrane implying that interactions between Texas Red and the membrane are energetically dominated. This shows that the specific design of

both, anchor molecules and probes, can be used to design specific orientational preferences. Importantly, they reveal that seemingly minute changes in molecular structure significantly alter the orientation with respect to the surrounding environment [66].

Most fluorophores are head group labeled but there are a few tail labeled systems. A homologous series of fluorescent 7–nitrobenz–2–oxa–1,3–diazol–4-yl (NBD)–labeled fatty amines of varying chain length (NBD–Cn) was studied in a POPC bilayer [67]. The NBD group prefers the hydrophilic–hydrophobic interface and hydrogen bonds with the lipid ester. The longer chained probe molecules prefer to locate close to the bilayer center and they can interdigitate between leaflets. This slows down their diffusion and increases coupling between the bilayer leaflets. The structure and dynamics of the POPC lipids in the neighborhood were weakly affected. These simulations agree with experiments from the same group and can explain them on a molecular level showing again the strength of atomistic simulations. The experiments showed a non–monotonicity for the photophysical parameters as well as the kinetic and thermodynamic parameters for their interaction with the POPC membranes [67]. For most lengths the probe disorders the lipid chains whereas the sodium counter ions of the charged probe bind to the lipid backbones and lead to an ordering effect. These two effects almost exactly cancel each other out leading to an overall weak disturbance. The NBD–16 is optimally length matched to the POPC and overall orders the system explaining the non–monotonic behavior as the others are weakly disordering. Compared to fatty amines acyl chain–labelled phosphatidylcholines, and sterols with the same tag, the chromophore in NBD–diCnPE locates in a similar region of the membrane but with a different orientation. This leads to an opposite interaction with the lipid dipoles and different electrostatic behavior [68].

Not only POPC but also DPPC bilayers with NBD acyl–chain labeled fluorescent analogs (C6–NBD–PC and C12–NBD–PC) have been studied by MD. The NBD fluorophore locates in this case transverse to the membrane and closer to the interface than to the bilayer center with the nitro group pointing towards the aqueous region. Hydrogen bonds form between the NH group of NBD and the glycerol oxygens and between the nitro oxygens of NBD to water. NBD rotates in faster than 5 ns in agreement with fluorescence anisotropy measurements [69, 70]. NBD labeled probes diffuse indistinguishably from DPPC [70].

Very few studies have investigated how changes in the external thermodynamic conditions influence the behavior of fluorescent lipids. In one such study, BODIPY–PC a tail labeled fluorescent lipid analog was studied in DPPC monolayers and bilayers at three surface pressures (3, 10, and 40 mN/m) and directly compared to experiments [54]. The MD simulations show that monolayer and bilayer systems behave essentially the same in terms of probe orientation and lipid order. Simulations and experiments agree that the tilt of the probe decreases with increasing surface pressure and the simulations can then characterize location and orientation to a degree not available in experiments. The simulations can again separate entropic and enthalpic effects showing that enthalpy dominates at high surface pressure and entropy at lower pressures, resulting in larger

tilt angles [54].

Often NMR is also used to understand lipid membranes [71, 72]. Most NMR experiments do not require labeling lipids. The standard experimental observable is the tail order parameter. The lipid tail order parameter, S_{CD} [73], provides a measure of the alignment of C–H (or C–D in case of deuterated lipids) bonds with respect to an external axis which is normally aligned with the bilayer normal. It is given by,

$$
\begin{aligned}
-S_{CD} &= \frac{2}{3}S_{xx} + \frac{1}{3}S_{yy}, && (1)\\
S_{\alpha\beta} &= \langle 3\cos\Theta_\alpha \cos\Theta_\beta - \delta_{\alpha\beta}\rangle, \qquad \alpha,\beta = x,y,z && (2)\\
\cos\Theta_\alpha &= \hat{e}_\alpha \hat{e}_z, && (3)
\end{aligned}
$$

where $\hat{e}_z$ is a unit vector in the chosen z-direction and $\hat{e}_\alpha$ is a unit vector in the local coordinate system of the tails. This quantity is accessible by NMR measurements and provides a useful means for quantitative comparisons between experiments and simulations.

In addition to normal lipids also the orientation of probes can be determined which allows an additional validation of the probe experiments. .

For example, in a combined ^{2}H–NMR and molecular dynamics study of pyrene in a POPC bilayer [37], the authors found very good agreement between experiments and simulations with regard to the orientation of pyrene in the bilayer. Inside the membrane, the pyrenes arrange parallel to the bilayer normal, i.e. they arrange along the lipids. In the simulations five pyrene molecules were added randomly in– and outside the membrane. They all quickly entered the hydrophobic region and placed close to the headgroups. No flip–flop between leaflets was was observed. To compare to NMR, S_{CD} was calculated for five distinct classes of bonds. Simulations confirmed that pyrene rotate along the bilayer normal and so in NMR only the rotational average along the long axis can be observed decreasing the signals. Simulations can as discussed determine all observables and their individual distributions. So all of the ordering tensor elements S_{xx}, S_{yy}, and S_{zz} could be calculated for each pyrene molecule. The average values lead to $S_{xx} = 0.33$, $S_{yy} = 0.07$ and $S_{xx} - S_{yy} \approx 0.26$, which is close to the NMR result of 0.25 showing that well designed atomistic models can determine and explain experimental data reliably.

Repáková et al. presented a very thorough study of the behavior and perturbations of DPH(1,6–diphenyl–1,3,5–hexatriene) in a DPPC bilayer [74, 75]. They employed 50 ns molecular dynamics simulations – which was very long at the time – to study the distribution, orientation, and dynamics of DPH fluorescent probes. DPH molecules stayed separate and do not prefer the membrane center where free volume would be largest but align with the hydrophobic acyl chains. There are a few flip–flops of DPH molecules from one leaflet to another in contrast to pyrene. The rotational diffusion time can be compared with experiments as well as the lateral diffusion of DPH in the plane of the bilayer. For lateral diffusion of DPH a jump diffusion mechanism between voids was found [74]. In a followup study the same group compared their simulations

with differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR) experiments to quantify the influence of DPH probes on the structure and dynamics of a bilayer. Atomistic MD simulations show that in the membrane–water interface the influence of DPH is minor, whereas in the acyl–chain region the DPH gives rise to major perturbations. DPH changes a wide range of membrane properties, such as the packing and ordering of hydrocarbon tails and the lateral diffusion of lipid molecules. The effects are prominent but as in most other local in nature, i.e., the changes observed in the properties of lipid molecules are significant in the vicinity of DPH, but reduce rapidly as the distance from the probe increases in agreement with DSC and NMR data. DSC shows only subtle perturbation to the cooperative behavior of the membrane system in the presence of DPH, and ^{2}H NMR shows that DPH gives rise to a slight increase in the lipid chain order [75].

3. EPR Probes

While fluorescent lipids are very widely used to understand lipid membranes also nitroxide spin probes are very useful to measure both orientation and motions in the range of 0.1 to 10 ns via EPR [31, 32, 33, 35]. Thus, lipids modified with nitroxide spin probes are particularly useful in describing membrane organization and dynamics. To date there is a limited number of simulation studies of spin markers in membranes available in the literature [76, 77, 78, 79, 80].

For EPR experiments paramagnetic probes are employed and most commonly nitroxide spin labels are used which contain a stable radical [30]. The EPR spectrum of the nitroxide depends on the nanosecond motions within the membrane making it an excellent method for probing local dynamics within domains of a bilayer [81]. The lineshapes in EPR reflect the internal dynamics; if the sample is properly aligned also the orientation of the probe can be measured by EPR spectroscopy. This alignment can be done magnetically [31, 32]. EPR experiments need a stable radical; a good example is S–(2,2,5,5–tetramethyl–2,5–dihydro–1H–pyrrol–3–yl) methyl methanesulfonothioate) (MTSL) which can be used for site directed spin labeling by attaching it chemically to relevant sites [82]. As in the case of all probes the analysis relies on the assumption that spin labels do not significantly alter the system behavior under study.

Kemmerer et al investigated the interaction between DPPC and a MTSL modified DPPC lipid. First the charge distribution on MTSL was determined by quantum chemistry and the label attached to a lipid similar to described above. The label position and its dynamics were then studied as well as the influence on the lipids in the neighborhood. Probe lipids are found to locate closer to the center of the membrane than regular lipids, i.e. they induce a local depression. This is in agreement with simulations on a different spin–label with labeling in the tail region [78] where the label stayed also in the hydrophobic region. The low density of the probe lipids does, however, not lead to an a overall change of the membrane thickness in at least partial agreement with experimental data on a similar headgroup labeled lipid [83]. The experiments

speak for a location of the headgroup close to the interface but it is not conclusive how close this localization is exactly. The spin label in the simulation is essentially at the interface and can rotate freely. The spin label does, however, not orient randomly as preferred orientations are found. The spin–label locally disorders lipids as the order parameter is clearly depressed for tails within 1 nm; this particularly applies to the first four carbon atoms in the tail. In this particular simulation there was no difference in diffusion to the unmodified lipids [80]. Diffusion data from an EPR experiment should still be used with care. In order to study the diffusion in more detail longer simulations and/or simulations at higher concentrations would be useful.

Stimson studied the EPR active nitroxide spin labels attached to stearic acid in DPPC bilayers [78] with a focus was on the effect of chirality and on ionization of the carboxyl group of the label. For a non–ionized species, the labels can flip–flop between the leaflets of the bilayer. Such transitions have been previously observed only in very rare cases in molecular simulations [78]. As these simulations were not using a reactive or polarizable force field the effect of pH was indirectly implemented by different ionization. In reality at a given pH particularly close to the pKA a distribution of the different charge states would be found in experiments. The data for uncharged and charged SASL are drastically different. The uncharged version is located further away from the headgroup and can flip–flop to the other leaflet. The incorporation of SASL into the lipid bilayer results only in a small increase in order parameters and a related decrease of surface area, but it does not change the overall bilayer structure. The authors chose a very high concentration of 11 mol % which is about an order of magnitude higher than typical in experiments. As they only found small perturbations it is safe to assume that at weaker concentrations no large effects are expected particularly as clustering was not observed.

Vartorelli et al studied the effect of the insertion of spin–labeled n–doxyl–stearic acid (n–SASL, $n = 5, 12, 16$) on the structure and dynamics of a gel phase to obtain depth profiles and configurations of the labeled molecules. The shortest SASL keeps its label at the water lipid interface, and the head group hydrogen bonds both to neighboring lipids and to the solvent. The longest SASL paramagnetic label sits at the lipid-lipid interface. For intermediate lengths the label has two different configurations at higher lateral pressure. The insertion of the labeled molecules at low enough concentrations (0.36 mol %) do not perturb global properties like area per lipid, tilt angle, or order parameters. Perturbations are again confined to a 1 nm neighborhood around the spin label in agreement with most other studies [79].

Relevant experiments can mark different parts of the stearyl molecule with a label to get information on different positions within the membrane. This is only correct if one assumes that the molecular orientation of the probe is independent of the labeling. There is indirect evidence for this through collision rates of the labels [84, 85, 86]. The simulations clearly show that on average labeled fragments are found at specific membrane depths but not every label can be mapped one to one to a unique position in the membrane. The width of the vertical position of the labels is about 0.3 nm in agreement with neutron

diffraction data [87]. The effect of chirality of the labels was not very pronounced but it cannot be excluded that there are small influences. This has not been studied further by any group yet.

Another EPR relevant family of spin labels includes perifosine and its synthetic spin–labeled alkylphospholipid analogues. Such molecules arrange randomly in water but as soon as they are in the asymmetric neighborhood in the membrane they adopt specific orientations and conformations with the alkyl chains pointing into the hydrophobic core and the charged groups located at the interface. These molecules are lipids analogues as they are amphipathic and they make good EPR targets. Such a doxyl group aligns with the interface even if it is in the middle of the chain preventing the fully extended conformation. For molecules which have their doxyl group at the end of alkyl chain, the spin–label arranges more slowly as the extended conformation rotates less easily [77]. This again shows that designing probes for specific tasks or specific time scales allows the test of different static and dynamic questions.

EPR line shapes can fundamentally be calculated from a Liouville equation assuming a stochastic reorientation of the spin probe [88, 89]. To challenge or confirm the assumptions behind such an approach MD can be used to generate the stochastic input to the Liouville equation. Hakannson followed this approach and calculated order parameters, reorientation dynamics and direct EPR data for spin labeled molecules as well as unmarked DPPC lipids for comparison. The timescale of the electronic spin relaxation for a label at carbon six of DPPC is about 12×10^7 rad s^{-1} and for an unperturbed DPPC $3 - 4 \times 10^7$ rad s^{-1} [76]. So it turns out that the dynamics of the spin–labelled lipids is about 3–4 times slower than bulk DPPC and the EPR line shape of the labeled ones is slightly broader. As in that study charges on the labels were omitted this slowdown is likely a lower bound as charges likely lead to electrostatic binding. This study shows that it might be justified to compare the spin–probe dynamics with the reorientation of DPPC molecules with an appropriate defined MD model but the quantitative comparison has to be taken with caution.

4. Modeling of Fluorescence Quenching by Spin Labels

Determination how deep different molecules penetrate into a biomembrane can help understand membrane structure and protein-lipid interactions. Experimentally this can be determined by fluorescence quenching. Spin–labeled lipids are commonly used as fluorescence quenchers in depth–dependent quenching where they are added in addition to fluorophores, i.e. one has two different probes where one inhibits the other [90, 91]. Here one of the attached chemical groups have an unpaired spin typically from a stabilized radical, these are the same types of molecules used as in EPR labeling experiments (see below). Simulations are often used in tandem with such quenching experiments to accurately calculate the depth of the fluorophore and the spin–label as extracting quantitative information from experimental quenching data is complicated because there are not many experimentally available quenchers and thermal disorder

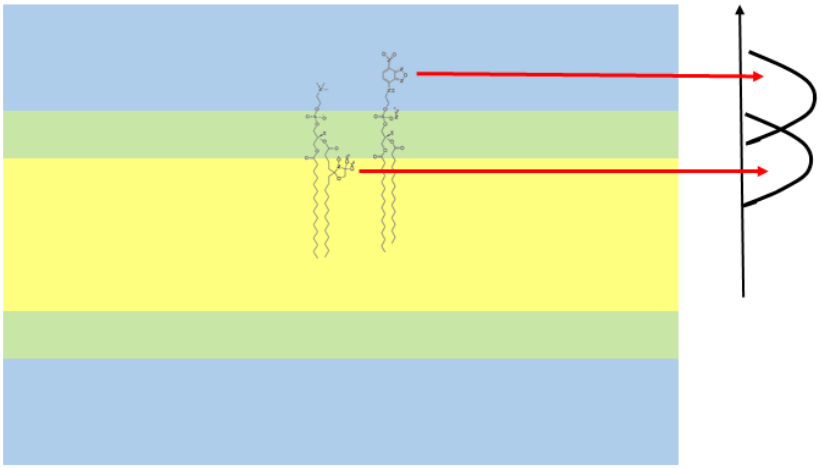

Figure 4: Left: Interaction of fluorophore and quencher in the membrane. Blue indicates water, green the headgroup region of the membrane and yellow the hydrophobic core. The left lipid is the doxyl quencher, the right a headgroup NBD labeled lipid following [92] Right: Sketch of depth profiles, the overlap of the curves are the quench region.

results in broad distributions of both quenchers and fluorophores as illustrated in Fig. 4.

MD has been used to study depth distributions of spin–labeled phospholipids in POPC bilayer. To probe different depths within the bilayer five different Doxyl–labeled lipids have been investigated, where the spin–label moiety was covalently bound to different carbon atoms (positions $n = 5$, 7, 10, 12, and 14) of the sn–2 chain of the phospholipid [90]. The spin probes turn out to be broadly distributed across the membrane with heterogeneous neighborhoods but in general the labeled carbons are at similar depths as their unlabeled counterparts at the same chain position. A broader and more heterogeneous distribution was found for a headgroup–attached Tempo spin label which due to its hydrophobic nature was deeper in the membrane than unlabeled headgroups. Depending on the concentration of Tempo–labeled lipids, the depth of the Tempo moiety was around 14 to 18 Å from the membrane center [90]. Comparison of the MD–estimated depths with the experimental suggestions allow to determine potential sources of error in depth–dependent fluorescence quenching studies [90].

For fluorescence quenching not only the spin label position but also the fluorescence probe need to be investigated. To that end, Kyrychenko developed and validated a general approach to determine the location of a fluorescent probe along the bilayer normal from quenching data. They used simulations of tryptophan octyl ester (TOE), in a POPC bilayer. The TOE ring locates in a broad maximum (almost 1 nm wide) about 1.5 nm from the bilayer center. Based on joint positions of quenchers and fluorophores quenching profiles can be determined. In an example the carbon atoms of the acyl chains of POPC were used as

pseudo-quenchers, i.e. no real spin–label was simulated. Then transverse overlaps and collision rates of the "quenchers" with TOE were determined. These simulated quenching profiles could be fitted by Gaussian profiles [93]. Comparison of these profiles with actual profiles of the indole moiety of TOE allows for testing of the validity of the data analysis and identification of the possible sources of error [91]. Error sources identified include the shielding of some deep lying quencher molecules by the acyl tails. There is also a variation in the depth of quenchers close to the probe due to local structural influences. Simulations have in this case the advantage that the full distribution of positions and orientations can be measured and not only averages where such subtle changes might be overlooked. The simulations also elucidated that water can penetrate to some extent into the system which often is neglected in the analysis of the experiments where in many cases just a simple slab model is used. Simulations can determine the complete density profiles and are able to elucidate the local water density close to the quenchers and probes.

Also the immersion depth of head–group labeled NBD in a POPC bilayer can be determined using such a technique. The immersion depth was estimated from fluorescence quenching with spin–labeled lipids in lipid vesicles where now directly the interaction between two different labels needed to be investigated. Six different spin–labeled lipids were used one with a headgroup–attached Tempo probe and five with chain–labeled Doxyl. The immersion depth and the width of the transversal distributions of the NBD moiety were estimated to be 15 and 6 Å from the bilayer center. This position agrees very well with MD simulations of NBD–PE in POPC (14.4 Å) [92].

5. Conclusions and Lessons Learned

There are a few things which became apparent over the last two decade or so in which mainly atomistic simulations have been used to explain and describe the behavior of probes in lipid bilayers. It is absolutely essential to perform such computational studies as experiments are highly specialized and yield great results but often the experiments are blind for the bulk of the lipids. Probe molecules are the few light bulbs in a sea of darkness.

The good news is that in general the perturbations of small probes are locally contained to the immediate neighborhood such that the overall behavior of the system is not strongly affected. This still does not absolve us from trying to understand these systems in high detail. The neighborhood of a probe often is structurally disordered which manifests itself by a locally larger area per molecule and lower order parameter. These structural differences are in most cases not homogeneously distributed over the thickness of the membrane but e.g. the order parameter is often affected at different carbon positions differently. A particular type of disturbance is binding of the probe to unlabelled lipids. This can lead to cluster formation and to changes in phase behavior. Only slight temperature shifts have been suggested up to now. As experiments are targeting more and more complex systems up to real cells simulations will need

to follow as the danger of strong preferential binding to other lipids or changes in conformations become more likely and potentially more detrimental.

Some studies particularly of free probes, i.e. not chemically bound to a lipid, like pyrene or DPH have shown that the probes can transfer spontaneously between leaflets. However, if as in a real membrane the two leaflets have distinctly different concentrations it becomes important to understand which leaflet is marked as e.g. diffusion measurements will depend strongly on this. This is one of the areas where further studies would be very useful.

A few studies have started to investigate what happens if we not only have one label per membrane or leaflets (as close to infinite dilution as one get get) but when there is an actual finite concentrating of probes or probe lipids. Most studies find that there is no significant cluster formation such hat the picture of the probe randomly intermixed is not far from reality. But large scale studies of free energies of binding among probes and between probes and lipids as a function of concentration are lacking. Very rare are currently also studies which target the interplay of different probes in the same system as is experimentally used in fluorescence quenching. The existing studies support the picture of effectively linear superposition but more studies are needed.

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