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Published in:
Journal of Physical Chemistry B

DOI:
10.1021/acs.jpcb.9b04967

Publication date:
2019

Document version
Accepted manuscript

Citation for published version (APA):

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Download date: 01. May. 2021
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J. Phys. Chem. B, Just Accepted Manuscript • DOI: 10.1021/acs.jpcb.9b04967 • Publication Date (Web): 05 Aug 2019

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Computational Characterization of a Cholesterol Based Molecular Rotor in Lipid Membranes

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Abstract

Biophysical properties of cellular membranes critically depend on their content of cholesterol and its interaction with various other lipid species. Cholesterol-dependent friction at the nanoscale can be studied with molecular rotors, whose quantum yield depends on rotational dynamics of functional groups during their excited state lifetime. Here, we present a detailed computational analysis of a phenyl-BODIPY linked cholesterol based molecular rotor in direct comparison with the well-known TopFluor-cholesterol. We describe a new parameterization strategy of force field parameters for the BODIPY moiety and carry out extensive molecular dynamics simulations of the probe in membranes in the absence or presence of cholesterol. Our study quantifies the extent of membrane perturbation by these probes, analyzes their tilting resistance in the bilayer and derives dynamic properties directly related to the rotor propensity. We show that phenyl-BODIPY-cholesterol bears the potential as a cholesterol-dependent molecular rotor to report about microviscosity of sterol-containing model and cell membranes.
Introduction

Cholesterol is an essential lipid component of the plasma membrane (PM) of mammalian cells. Its ability to order the acyl chains of phospholipids and thereby condense the lipid bilayer is instrumental for cholesterol’s propensity to regulate membrane permeability, bending flexibility, and lateral diffusion.\textsuperscript{1,2} When mixed with two phospholipid species, one bearing saturated and the other unsaturated fatty acyl chains, cholesterol can mediate the coexistence of two fluid phases, one called the liquid disordered (ld) and the other the liquid ordered (lo) phase. The cholesterol-rich lo phase has similar properties as lipids in the PM, while the ld phase resembles properties of lipids in the endoplasmic reticulum (ER).\textsuperscript{2,3}

Cholesterol’s distribution between cellular membranes is very heterogeneous with a high enrichment in the PM and recycling endosomes, intermediate levels in the Golgi and in endolysosomes but very low levels in the ER, where the biosynthetic machinery for cholesterol synthesis and feedback regulation of its cellular abundance reside.\textsuperscript{2,3} Membrane-embedded proteins sense and respond to the levels of cholesterol in each organelle, and a major question is, which molecular mechanisms underlie such sterol sensing processes. One mechanism is the direct binding of cholesterol to a particular protein. This has been shown for G-protein coupled receptors in the PM or for several ER resident proteins, like the transcription factors sterol response element binding protein (SREBP) and its binding partner INSIG,\textsuperscript{4} hydroxymethyl-glutaryl-CoA reductase, catalyzing the rate-limiting step in cholesterol synthesis or acyl-CoA acyl-transferase (ACAT), which esterifies cholesterol for storage as cholesteryl esters in lipid droplets.\textsuperscript{4,5} Also, via its ability to condense the lipid membrane, cholesterol can exert a more indirect effect on bilayer properties by altering local membrane packing and viscosity. This effect has been studied experimentally and in simulations using model membranes harboring reconstituted membrane proteins,\textsuperscript{6,7} but it is little characterized in intact cells. The latter is mostly a consequence of technical challenges in monitoring local membrane properties on spatial scales smaller than the resolution limit of optical microscopy as being used in live-cell imaging of appropriate cholesterol analogs.\textsuperscript{5} Cholesterol-
induced changes of membrane properties in living cells are often addressed indirectly, e.g., by following the trafficking itineraries and kinetics of selected cargo molecules\textsuperscript{9,10} or by using ratiometric measurements of fluorescent probes sensing alterations in packing density\textsuperscript{11}.

One alternative approach to overcome the limitation of diffraction limited imaging is to use membrane-embedded fluorescent molecules, whose quantum yield depends on the microviscosity of the surrounding membrane environment. Such so-called molecular rotors have been designed based on several lead structures including meso-substituted boron-dipyrrin (BOD-IPY) coupled to a phenyl group and attached to lipids, such as phosphatidylcholine, fatty acids, cholesterol or a farnesyl group\textsuperscript{12-15}.

Fluorescent analogs of natural lipids have to be carefully characterized since the covalently linked fluorophore can significantly affect the physicochemical and biological properties of the lipid molecule. This problem has been well-documented for analogs of cholesterol, where even minor modifications can profoundly affect the features of the parent sterol molecule\textsuperscript{16,17}.

Attaching a BODIPY group either in meso- or para-position to the short aliphatic side chain of cholesterol via an ester linkage was pioneered by Bittman and co-workers and was shown to generate cholesterol analogs with reasonable resemblance of the natural sterol\textsuperscript{18-24}. The analog with BODIPY attached in its meso-position has been commercialized as TopFluor-cholesterol by Avanti Polar Lipids Inc. (Alabama, USA) and has since been used in many cell biological and biophysical applications (reviewed in\textsuperscript{25,26}). This cholesterol analog is transported between PM and recycling endosomes with comparable kinetics as the intrinsically fluorescent cholesterol mimics dehydroergosterol (DHE) and cholestatrienol (CTL), has been used as a faithful probe for measuring cellular cholesterol efflux and was found to have similar partitioning into liposomes of differing phospholipid species as CTL or cholesterol\textsuperscript{22,27,28}.

Finally, although TopFluor-cholesterol is excessively targeted to lipid droplets in cells with high-fat content and is in itself not able to exert a membrane condensing effect in lipid bilayers\textsuperscript{22,29}, it partitions with some preference into the biologically relevant and cholesterol-rich $\alpha$ phase in model membranes\textsuperscript{19,21,24}.

Extending the linker between the BODIPY group and
cholesterol was for several analogs shown to diminish the resemblance of cholesterol. Thus, minor differences in how a dye is attached to the sterol backbone can have substantial consequences for the properties of the generated cholesterol probe.

Molecular rotors based on BODIPY attached to cholesterol have the potential to report about micro-viscosity of cholesterol-rich membranes. Two such probes have been recently introduced; one in which a phenyl-BODIPY rotor was attached to cholesterol’s side chain via an ether linkage and the other, in which an alkyl linker was used. The first probe has been shown to report about micro-viscosity in model membranes but failed to partition into lo domains in ternary model membranes. The membrane properties of the second rotor probe have not been studied yet.

Here, we have carried out a computational analysis of the molecular membrane properties of this second phenyl-BODIPY-cholesterol rotor in direct comparison to TopFluor-cholesterol. We perform a set of MD simulations in model membranes with or without cholesterol to quantify i) how large a perturbation the cholesterol-linked probes cause to the properties of the native membrane, ii) the orientation and placement of the cholesterol linked probes, and iii) the rotor function of the phenyl-BODIPY probe. Such a study requires careful parameterization of the force-field parameters for the BODIPY moiety, which cannot be inferred from standard force-fields. To derive such parameters used later in classical molecular dynamics (MD) simulations of the BODIPY-tagged cholesterol analogs, we used the method of force-matching, by which we tailor our force-field to reproduce specific quantum chemical reference data. Thus, we develop a force-field that provides high-level structural results and allows for running MD simulations on long time-scales. The basic idea used in force-matching procedures is to minimize the residual between forces calculate from a force-field and from accurate, but expensive reference forces calculated with an ab-initio method. The force-matching method has been used extensively with one of the more recent and widely accessible and sophisticated implementations being the ForceBalance program. Use of the force-matching approach to derive a given force-field is much more advanced com-
pared to e.g., regular fitting of the electrostatic potential\textsuperscript{15,20,47} and we will, therefore, as part of this study, present results of a set of computational tests which validate the quality of the derived force-field parameters for simulation of fluorescent probes in membranes.

**Computational Details**

**Parameterization of the BODIPY probes**

Force-field parameters of the phenyl-BODIPY and TopFluor probes were created and systematically optimized based on quantum-chemical reference data using the ForceBalance program of Lee-Ping Wang.\textsuperscript{42} For the functional form of the force-field, an AMBER-like description was employed

\[
E = \sum_{\text{bonds}} \frac{k_b}{2}(b - b_0)^2 + \sum_{\text{angles}} \frac{k_{\theta}}{2} (\theta - \theta_0)^2 + \sum_{\text{dihedrals}} k_{\phi}(1 + \cos(n\phi - \gamma)) + \sum_{i<j} \frac{q_i q_j}{r_{ij}} + \sum_{i<j} \left( \frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}} \right)
\]

(1)

This functional form consists of bonded terms (bonds, angles, dihedrals) and non-bonded terms (charges, Lennard-Jones (LJ)). In the course of the force-field derivation, we only optimize the bonded parameters explicitly. The charges were updated separately by fitting them to the molecular electrostatic potential (ESP). The LJ parameters are left unmodified. This separation into intra- and intermolecular degrees of freedom is appealing since it simplifies a difficult optimization problem into two smaller and easier ones. Further, the energies/forces will be computed from vacuum calculations, and so only represent a good source of reference data for the degrees of freedom relating to intramolecular degrees of freedom. Fitting to ESPs is a well-known and successful technique to obtain high-quality partial charges for molecular force-fields, based on the idea of selecting charges that reproduce, as well as possible, the quantum mechanical ESP on a molecular surface.

Due to computational efficiency, the parameters were derived for smaller molecular sub-
units of only the BODIPY unit and a linker group, instead of on the much larger cholesterol-linked fluorophores. We denote this smaller unit the unlinked BODIPY probes. The structures are shown in figure 1 along with the structures of the final cholesterol-linked molecules to be used in the MD simulations.

Parameter optimization was carried out on the isolated unlinked subunits, and final parameters of the cholesterol conjugated dyes were assembled based on these parameters along with existing cholesterol parameters from the lipid14 force-field. The unlinked BODIPY units used in the optimization were constructed with a sufficiently long alkyl chain linker such that parameters from at least one of the force-fields (force-matched FF or lipid14) would be available. Initial values for each parameter were taken from GAFF where possible (any parameter not involving the boron atom). The GAFF force-field contains no parameters related to the boron atom in BODIPY, so these were instead taken from the OPLS_2005 force-field. An initial set of charges were assigned according to the RESP procedure using the Antechamber program based on ESPs computed at the B3LYP/6-31+G* level of theory.

With this initial set of parameters established, an iterative procedure was set up, as shown in figure 2. First, the classical force-field was used with an MD program as a generator of structures. Second, the structures from the classical MD were used for evaluation of energies and forces with a quantum chemical method. Finally, the force-field parameters were updated according to these new reference data. This procedure was carried out for 10 iterations, after which the force-field parameters did not change significantly.

MD simulations intended to generate equilibrium structures were carried out for the probes in isolation with no cutoffs, using the Gromacs program, version 2018.2. A 1-fs time step was employed, and the system was propagated using stochastic dynamics for a total of 1 ns. The target temperature was set to 300 K and the friction constant $\gamma$ was set to 0.5 ps$^{-1}$. Structures were sampled every 10 ps, for a total of 100 structures in each iteration.

The QM energies and forces were evaluated using Orca, version 4.0.1.2, at a B3LYP/def2-SVP level of theory. The RIJCOSX approximation was employed with the def2/J auxiliary.
iliary basis set to reduce computational effort. Electrostatic potentials were evaluated on molecular surfaces using the \texttt{orca_vpot} utility, and an ESP fit was carried out to update the charges. The surfaces used in the fit were generated according to the algorithm of Saff and Kuijlaars,\textsuperscript{55} with the radii of the spheres scaled according to the van der Waals radii of the atoms. Two surfaces were used in each structure, with scaling factors of 1.4 and 2.0, corresponding to regions of typical intermolecular interaction. In the ESP fit, symmetry-equivalent atoms were constrained to have the same charge, and a non-redundant fitting scheme realized a total molecular charge of zero. The charges are obtained by minimizing the sum-squared error between the reference QM ESP and the approximate ESP generated by the charges across all the structures of the current and all previous iterations. We used the SLSQP\textsuperscript{56} minimization routines available in SciPy\textsuperscript{57} for the practical minimization procedure.

We optimized the force-field parameters with a Newton-Raphson optimizer from the ForceBalance\textsuperscript{42} program package. A regularization prefactor of 0.01 was used, along with an initial trust radius of 1.0 in the mathematical parameter space. Structures, and associated energies and forces, from both the current and all previous iterations, were included in the target cost function. As a result, the reference data set grows linearly in size with the number of iterations. The resulting force-matched force-field will be denoted FBFF in the following sections.

**Molecular dynamics simulations in membranes**

For the membrane simulations, initial membrane configurations were constructed using the CHARMM-GUI\textsuperscript{58} web server. Membrane systems consisting of 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) (which is a lipid composed of a phosphatidylcholine (PC) head group, and palmitoyl (PA) and oleoyl (OL) tails) and cholesterol (CHL) were prepared with either pure POPC or as a mixed 30% MOL cholesterol/POPC membrane. Three variants were prepared in each case, containing either no probe, a single TopFluor-cholesterol probe or a single phenyl-
Figure 1: Molecular structures of the two cholesterol-BODIPY probes (termed linked) along with the subunits used in the optimization procedure (termed unlinked). Also highlighted the different subregions in the molecules, including the BODIPY units (purple), phenyl ring (orange), linker (blue), and cholesterol (green). The atoms of the dihedral angle responsible for the rotor function in phenyl-BODIPY (CB19-CB20-CB21-CB22) are highlighted.
Figure 2: The workflow used for the force-field optimization. Starting from an initial set of force-field parameters, a classical MD program is used to generate a trajectory of structures. These are fed into a QM program, which computes energies, forces and electrostatic potentials. This is then used as reference data with a force-field optimization procedure, and an updated force-field is derived, which is fed back to the MD driver to generate a new trajectory. The process is repeated until convergence.

BODIPY-cholesterol probe. The POPC membrane was constructed with 91 POPC lipids in each leaflet, while the 30\% MOL membrane was constructed with 27 cholesterol and 64 POPC lipids in each leaflet. In addition, we also included a pure POPC membrane with just a single, untagged cholesterol molecule, using the same protocol as was used for the remaining membrane systems. In each case, 9100 TIP3P water molecules were added to the membrane, corresponding to a hydration number of 50 waters to each lipid component. Physiological salt conditions were generated by including 0.15M KCl in the simulation box. In the probe-containing systems, the periodic box was expanded in the $x$-direction, and the probe in question was placed in the (temporary) vacuum along the edge of the box.

Following this initial construction, all systems were equilibrated in a multi-stage procedure, after which 900 ns long MD simulations were carried out. The initial step of the equilibration procedure consisted of a steepest-descent minimizer which was applied at constant volume for 5000 steps to remove any bad contacts. Next, the systems were brought
to a temperature of 303.15 K across 50 ps. A time-step of 1 fs was adopted, and the target
temperature was reached using the Berendsen thermostat. Following this, the density of
the system was equilibrated in two stages, first for 25 ps using a 1 fs time-step, followed by
a 300-ps run using a 2 fs time-step. Throughout the equilibration steps, the pressure was
controlled towards 1 bar using a semi-isotropic Berendsen barostat, with a time constant of
the pressure coupling of 5.0 ps and a compressibility of \(4.5 \times 10^{-5}\) bar\(^{-1}\).

The systems were finally equilibrated using production settings for 100 ns, followed by a
final 900 ns production run. The production MD was run in an NPT ensemble, using a Nose-
Hoover thermostat \(^{61,62}\) with a time constant for the temperature coupling of 8.0 ps towards
a reference temperature of 303.15 K. The pressure was controlled towards 1 bar with a semi-
isotropic Parinello-Rahman barostat \(^{63}\) The time constant of pressure coupling was kept at
16.0 ps, and the compressibility was set to \(4.5 \times 10^{-5}\) bar\(^{-1}\). Bonds involving hydrogens
were restrained using the LINCS algorithm \(^{64}\) Long-range electrostatics were treated using
the Particle Mesh Ewald method, with a short-range cutoff of 12 Å.

**Force-field validation**

We examined the quality of FBFF for the BODIPY probes by comparing the geometries and
vibrational frequencies computed with either the force-field or with the same QM method as
was used in the derivation (B3LYP/def2-SVP) of the force-field. We include in this analysis
also a GAFF-like force-field, which contains standard GAFF parameters for all bonds, angles,
and dihedrals. Any parameters involving boron were carried over from the FBFF, as GAFF
does not cover parameters relating to the boron atom. Both the final cholesterol-linked
structures as well as the unlinked units used in the force-field optimization procedure were
included in the validation. For the energy minimization, Hessian construction and normal
mode evaluation using the force-field, the double-precision variants of the Gromacs \texttt{mdrun}
and \texttt{nmeig} programs were employed. Second, the degree to which QM energies and forces
(from the training data) can be reproduced with the force-field was also investigated. This
was done with the single-point evaluation mode available in ForceBalance. Finally, the average bond length and angles extracted from a 100 ps \textit{ab-initio} molecular dynamics (AIMD) B3LYP/def2-SVP trajectory in the NVT ensemble was compared to an equivalent 100 ps classical MD trajectory computed using the force-field. The Terachem program, version 1.9.3, was used to run the AIMD. A Bussi-Parrinello Langevin integrator with a time-step of 1 fs was employed, and structures were saved every time step. Equivalent simulations in the NVT ensemble were set up for the force-fields using Gromacs. Average bond and angle parameters were then computed for each trajectory.

**Analysis of membrane properties**

Determination of the area per lipid and membrane thickness was performed using the FAT-SLiM\textsuperscript{65} program. The head-groups were defined from the phosphorous atoms of POPC lipids and the oxygen atom of cholesterol molecules.

Order parameters are calculated according to

\[ S_{CH} = \langle 3 \cos^2 \theta - 1 \rangle / 2, \tag{2} \]

where \( \theta \) is the angle between a lipid tail C–H bond vector and the membrane normal. \( \langle \cdot \rangle \) denotes an ensemble average. The calculations were based on eq. \( \text{[2]} \) with the help of the MDAnalysis\textsuperscript{66,67} python library. To compute local order parameters, the averaging over molecules in eq. \( \text{[2]} \) was restricted based on the projected distance (in the \( xy \)-plane) between the probe molecule and the lipid tails. The local order parameter was calculated binned into annuli. The position of the molecules was defined from the center of geometry (COG) The COG-COG distance was calculated, taking into account periodic boundary conditions. Error estimates were calculated by block averaging of the raw data \( \cos(\theta)^2 \) used as input to the ensemble average in eq. \( \text{[2]} \) using the gromacs analyze tool.

Tilt angles of cholesterol and the BODIPY units were computed using trajectory reading
utilities from the MDAnalysis\textsuperscript{66,67} python library.

Partial densities were computed using the Gromacs\textsuperscript{51} density program, with centering of the membrane bilayer based on the positions of the PC head-groups.

Dihedral angles (and auto-correlation functions (ACFs) thereof) were computed using the cpptraj program.\textsuperscript{68} The ACFs were fitted to analytical expressions with the non-linear fitting tool available in the grace program.\textsuperscript{69}

Results and Discussion

Validation of force-fields

To quantify the quality of FBFF, a comparison between the geometries of the force-field minimized structures, and the QM minimized structures provides a simple initial validation test. The force-field and QM optimized geometries of the unlinked probes are shown superimposed in figure\textsuperscript{3}A-B, while the cholesterol-linked probes are shown in figure\textsuperscript{3}C-D.

The root-mean-square deviation (RMSD) in the coordinates between the FF and QM optimized structures is reported in table\textsuperscript{1}

<table>
<thead>
<tr>
<th>Molecule/force-field</th>
<th>Unlinked</th>
<th>CHL linked</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TopFluor</td>
<td>phenyl-BODIPY</td>
</tr>
<tr>
<td>FBFF</td>
<td>0.054</td>
<td>0.088</td>
</tr>
<tr>
<td>GAFF</td>
<td>0.207</td>
<td>0.091</td>
</tr>
</tbody>
</table>

For the unlinked probes, FBFF has for TopFluor an RMSD of 0.054 Å, which is four times lower than with the corresponding GAFF force-field. For phenyl-BODIPY, the performance of the two force-fields is comparable, both having an RMSD relative to the QM reference of about 0.09 Å. For the cholesterol-linked probes, the RMSDs are in general larger, which is a consequence of having only optimized the parameters of the BODIPY unit. As with the unlinked probes, the RMSD is lower when using the FBFF, especially for phenyl-BODIPY.
Figure 3: Superimposed structures minimized using QM (green), FBFF (blue) or GAFF (red). The structures shown are of the unlinked probes phenyl-BODIPY (A) and TopFluor (B) and the cholesterol-linked phenyl-BODIPY (C) and TopFluor (D).
Overall, the performance of FBFF in reproducing minimum energy structures is seen to be acceptable.

After the structures have been minimized, vibrational frequencies and normal modes can be extracted from the diagonalized mass-weighted Hessian. The harmonic frequencies of the BODIPY probes, computed either with the QM reference or with a force-field, are shown in figure 4.

![Harmonic vibrational frequencies of the cholesterol-linked probe molecules and the unlinked BODIPY probes. The frequencies are plotted sorted by magnitude. The reference QM-derived frequencies were evaluated at a B3LYP/def2-SVP level of theory.](image)

For the unlinked probes, it is clear that both the GAFF and FBFF offer an excellent reproduction of the harmonic frequencies. With TopFluor, the GAFF force-field offers marginally better harmonic frequencies than FBFF. This situation is reversed for phenyl-BODIPY, where FBFF offers slightly better reproduction of the frequencies than GAFF, especially...
for the C–H stretch region above 3000 cm⁻¹, where the GAFF frequencies are somewhat underestimated compared to the QM reference. The reproduction of frequencies is poorer for the linked probes. Here, the medium-frequency range (500–1500 cm⁻¹) is in general underestimated by both force-fields. A striking deviation in both force-fields is seen with the O–H stretching vibration, which is severely underestimated: the reference QM calculation places this vibration at 3800 cm⁻¹, but no vibration at this position is to be found in either force-field. The cholesterol part of the linked probes is described by the lipid14 force-field in both cases. The parameters of the cholesterol itself are therefore not optimized against the B3LYP/def2-SVP QM reference, which is the reason for the large error in the O–H stretching vibration frequency. This is, however, not a major concern, as the force-field is typically used with constraints on bonds involving hydrogen, as is generally done for computational efficiency in biomolecular force-fields. Keeping the cholesterol parameters from lipid14 ensures a consistent description of all the lipid components when we later apply the force-field to MD simulations of membranes. Overall, for the reproduction of frequencies, we do not, in this case, see any significant improvements by using a force-matched force-field.

Reproduction of QM reference data (energies and forces) constitutes the input to the actual objective function used in the FF optimization procedure. Table 2 reports how well this is reproduced with the optimized and GAFF force-fields. The GAFF force-field has significantly higher errors in both the reproduction of the energies and the forces. For TopFluor, the error is reduced from 27.52 kJ/mol with GAFF to just 8.86 kJ/mol with the FBFF. GAFF performs better on phenyl-BODIPY with an error of only 15.08 kJ/mol. FBFF gives, as before, a substantial improvement over GAFF, reducing the error in the energy reproduction down to just 5.78 kJ/mol. With both molecules, the reproduction of the QM forces is also improved with FBFF, with the error in the reproduction of the forces being about 2.8 times lower.

As a final test, we compare average bond lengths and angles extracted from NVT MD trajectories from either a QM reference or using classical MD with a force-field. This is
shown in figure 5.

Figure 5: The average bond lengths and angles extracted from a 100 ps NVT AIMD trajectory are compared to the average bond lengths and angles using classical MD.

The bond lengths are very well reproduced by FBFF. The average mean absolute deviation (MAD) in the average bond lengths using FBFF (GAFF) force-field is 0.08 (1.15) pm for phenyl-BODIPY and 0.21 (1.73) pm for TopFluor. The average angles are slightly less impressive but are again better than with the GAFF description: the MAD in the angles using FBFF (GAFF) force-field is 0.15 (1.20) degrees for phenyl-BODIPY and 0.42 (1.96) for TopFluor. This test shows clearly the improvement in the quality of the internal structures.
by using FBFF.

Overall, we conclude that our approach of coupling classical molecular dynamics for sampling with force-matching for the force-field parameter optimization is a viable strategy to obtain high-quality, tailored force-fields. The most substantial improvements are seen in terms of the reproduction of energies and forces and equilibrium structural parameters extracted from NVT MD trajectories. Minimum-energy structures are also somewhat better with FBFF, but vibrational frequencies are not improved to any significant degree compared to a GAFF-like force-field.

Table 2: Average errors in energies and forces between the QM reference and the force-fields (GAFF or FBFF). The reference data from the last optimization iteration was used. The errors were evaluated with a single-point evaluation with the ForceBalance program.

<table>
<thead>
<tr>
<th>Observable/force-field</th>
<th>TopFluor</th>
<th>Phenyl-BODIPY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GAFF</td>
<td>FBFF</td>
</tr>
<tr>
<td>Total energy (kJ/mol)</td>
<td>27.52</td>
<td>8.86</td>
</tr>
<tr>
<td>Force (kJ/mol/Å)</td>
<td>134.47</td>
<td>48.46</td>
</tr>
</tbody>
</table>

**Bulk membrane properties**

Having validated the improved force field parameters of the cholesterol probes, we turn to examine the extent of perturbation caused by both BODIPY-tagged cholesterol analogs in lipid membranes. For this purpose, we performed MD simulations of the probes in two types of POPC model membranes: one with and one without cholesterol. A 30% MOL concentration of cholesterol was used in the former case. The membrane thickness and area per lipid are reported in table 3.

These properties are widely used as indicators of membrane structure and ordering. Comparing the cholesterol-containing membrane to the pure POPC membrane, we see that the inclusion of cholesterol leads to an increase in membrane thickness of roughly 0.3 nm, while the area per lipid decreases by 0.12 nm².

Looking at the membranes containing BODIPY-tagged cholesterol, we see that the bulk
Table 3: Membrane thickness and area per lipid for the POPC and 30% CHL/POPC model membrane systems. The area per lipid is computed on a per-component basis. Error estimates based on block averaging are listed in parenthesis.

<table>
<thead>
<tr>
<th>System</th>
<th>Thickness (nm)</th>
<th>Area per lipid (nm$^2$)</th>
<th>POPC</th>
<th>CHL</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>3.782 (0.00279)</td>
<td>0.681 (0.000650)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>POPC + phenyl-BODIPY</td>
<td>3.777 (0.00299)</td>
<td>0.682 (0.000694)</td>
<td>–</td>
<td>0.422 (0.00803)</td>
<td>–</td>
</tr>
<tr>
<td>POPC + TopFluor</td>
<td>3.778 (0.00299)</td>
<td>0.682 (0.000652)</td>
<td>–</td>
<td>0.418 (0.0120)</td>
<td>0.422 (0.00803)</td>
</tr>
<tr>
<td>30% CHL</td>
<td>4.105 (0.00491)</td>
<td>0.553 (0.00112)</td>
<td>0.392 (0.00119)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>30% CHL + phenyl-BODIPY</td>
<td>4.092 (0.00472)</td>
<td>0.555 (0.00109)</td>
<td>0.394 (0.00155)</td>
<td>0.382 (0.0344)</td>
<td>–</td>
</tr>
<tr>
<td>30% CHL + TopFluor</td>
<td>4.094 (0.00385)</td>
<td>0.556 (0.00107)</td>
<td>0.392 (0.00195)</td>
<td>0.403 (0.0179)</td>
<td>–</td>
</tr>
</tbody>
</table>

Properties of the membrane are virtually identical to that of the parent membrane. For example, we computed an average area per lipid of the POPC lipids in a POPC membrane of 0.681 nm$^2$, which largely unchanged upon adding either a cholesterol-linked phenyl-BODIPY or TopFluor probe, both having a computed area per lipid of 0.682 nm$^2$. The area occupied by the cholesterol-linked membrane probes is similar to that of native cholesterol (0.392 nm$^2$) for both phenyl-BODIPY (0.382 nm$^2$) and TopFluor (0.403 nm$^2$). The error estimates associated with the area per lipid of the probes are higher than the error estimates of the native cholesterol due to the reduced sampling associated with going from 54 copies of cholesterol to just a single molecule (we can roughly expect a factor of $\sqrt{54} \approx 7.3$ higher errors). For this reason, it is difficult to judge whether the observed differences in the area occupied by the tagged cholesterol probes are significant.

With the size of membranes used, adding a single BODIPY linked cholesterol corresponds to a 0.5% concentration on a molar basis. This is in the range of values used in the labeling of cellular membranes for live-cell imaging of sterol transport, ensuring that this little amount of probe does not affect bilayer properties.\(^{22}\)
Order parameters

Another common way of investigating membrane structure is through the order parameters, $S_{CH}$, of the lipid tails. These inform about the membrane structure by quantifying the degree of ordering of the C–H bond vectors of the lipid acyl chains. We observe no significant differences in the order parameters on a global level for a particular membrane composition (cholesterol-poor or cholesterol-rich) upon including the cholesterol-linked probes. Therefore, we turned to look at the impact of the local structure, as shown in figure 6, by restricting the average over molecules in eq. (2) based on the distance between a lipid tail and the probe molecule.

In the membranes containing BODIPY probes, in regions far away from the probe (more than 20 Å), the calculated order parameters coincide with the bulk order parameters calculated for the corresponding probe-free membranes. In a medium-range regime of 10–20 Å there are some minor visible differences, but it is only at short range that differences are significant. We, therefore, focus on this innermost region.

For the TopFluor probe, the order parameters of the lipid tails in the immediate vicinity (within 0–5 Å) are significantly lowered. The effects are most pronounced for carbon segments in the lower end of the tail (C10-C18 in OL, C8-16 in PA). In the cholesterol-containing membrane, the average order parameter of carbons 10–18 in the OL tail decreases from 0.1915 to 0.1717. These reductions are significantly larger than the maximal estimated error bars in the region, which are always less than 0.0093. Meanwhile, in the PA tail, the order parameters of carbons 8–16 decrease on average from 0.2640 to 0.2356. Again, the reductions are significant, considering the maximal estimated error bars of 0.0103.

In the POPC membrane, the same trends in the average order parameters are seen for the OL carbons 10–18 (decrease of 0.1279 to 0.1088) and PA carbons 8–16 (decrease of 0.1829 to 0.1656). One curious feature is apparent for OL carbons 2–6 in the POPC membrane, where the associated error bars are significantly larger than in any other case. Here, the estimated error becomes as large as 0.036. As we will later see, this larger error
Figure 6: Local lipid tail order parameters $S_{CH}$ of the sn2/OL (left) and sn1/PA (right) for the POPC and 30% CHL membranes, with or without including BODIPY probes. The spatial averaging is restricted to be within concentric rings of the probe – lipid distance in the $xy$ plane.
can be partially understood due to the presence of two very long-lived orientations of the cholesterol-TopFluor probe, see figure 7, one in which the tilt angle of the BODIPY unit is near 150 degrees (100–400 ns), and one in which it fluctuates more freely. Considering only the first half of the trajectory leads to a smaller error estimate (0.022).

The phenyl-BODIPY probe has similar effects as the Topfluor one in the cholesterol-containing membrane, albeit with a smaller magnitude in the decrease in the order parameters. Again, the changes are localized to carbons 10–18 in the OL (average decrease from 0.1904 to 0.1799) and carbons 8–16 in PA (average decrease from 0.2644 to 0.2541). In the pure POPC membrane, the changes are of the opposite sign and much smaller in magnitude.

We observe on average a slight increase in the order parameters (PA: 0.1845 to 0.1827, error estimate 0.0071; OL: 0.1270 to 0.1351, error estimate 0.0068) in the immediate vicinity of the probe. One should keep in mind that the observed differences in average order parameters are here on the same order of magnitude as the error bars.

Overall, while the order parameters at a global scale are unaffected by the inclusion of membrane probes, there is a definite impact on the ordering of the membrane at the local level. These local effects are, for both probes, relatively small compared to the global changes in the order parameters when going from, e.g., a pure POPC membrane to a 30% MOL cholesterol/POPC membrane. For example, comparing the order parameters of C14 of the OL tail in a POPC membrane (0.1419) to a CHL membrane (0.2053), we can see much larger changes in the order parameters than what was induced locally by the probes.

**Tilt angles**

The extent of sterol tilting in the membrane relative to the bilayer normal is closely related to the ordering capacity of both cholesterol and its analogs. Sterol effects on membrane thickness, permeability, and elastic properties have been directly linked to the extent of sterol tilting in the bilayer, which is why we compared the extent of sterol tilt for cholesterol with those of its BODIPY-tagged analogs. Figure 8 shows the tilt angle of the cholesterol unit,
defined from the angle between the C10–C13 vector and the membrane normal, for both
standard cholesterol molecules and for the BODIPY tagged cholesterols.

Figure 7: Tilt angle of the BODIPY unit, defined as the angle between the shown vector in
the BODIPY unit (lower right panel) and the z-normal. The top left panel shows a trace
(light color), as well as the running average (solid) of the tilt angle of the two probes. The
bottom left panel shows histograms of the tilt angle. The four panels in the top right show
2D histograms over the CHL and BODIPY tilt angles.

The tilt angle distribution of native cholesterol is unaffected by the presence of the BOD-
IPY probes, with the maximum of the distribution being 13 degrees in all cases. Comparing
native cholesterol to the BODIPY tagged cholesterols in 30% MOL cholesterol, both probes
show broader distributions shifted towards higher angles. At first glance, the tilt angle distrib-
ution of the TopFluor tagged cholesterol resembles that of native cholesterol slightly better
than that of the phenyl-BODIPY tagged cholesterol. Considering the associated error bars,
however, such judgments should be done with care. Whereas there is good sampling with
only minuscule error bars for the native cholesterol in the 30% CHL membranes, much larger
error bars are present for the conjugated probes due to reduced sampling and more complex
dynamics. As such, it is not possible to observe significant differences between the conjugated
Figure 8: Tilt angle of the cholesterol. The tilt angle is defined as being between the C10–C13 bond vector and the membrane normal. The three black lines are almost exactly on top on each other. Also shown is two representative snapshots from the MD, namely of phenyl-BODIPY-cholesterol in the cholesterol membrane and Topfluor-cholesterol in the POPC membrane. The vector used in the tilt angle definition is shown in blue. Error bars were estimated from the standard error of the mean in the distributions by sub-dividing the trajectory into 45 blocks of equal length (2 ns).

cholesterol tilt angle distributions of the two probes in cholesterol-rich membranes.

In the POPC membrane, which is less ordered, the tilt angle distributions of both BODIPY-tagged cholesterols is shifted towards higher angles compared to the membrane containing 30% MOL cholesterol. The TopFluor tilt angle distribution is shifted towards higher angles than phenyl-BODIPY. To see which of these distributions have the largest similarity to native cholesterol, we compared the distributions to that of just a single cholesterol molecule in a pure POPC membrane. This tilt angle distribution was extracted from an MD simulation of a single cholesterol molecule in a POPC membrane, using the same MD protocol as was used for the remaining membrane simulations. The resulting distribution of the untagged cholesterol shows the largest resemblance to phenyl-BODIPY cholesterol. The most probable angle of native cholesterol and phenyl-BODIPY tagged cholesterol coincide at 21 degrees. For TopFluor tagged cholesterol, the most probable angle instead occurs at 37 degrees.
This is in contrast to the tilt angle distributions from the POPC membrane having 30% MOL cholesterol, where the two tilt angle distributions were similar, suggesting that the extent of sterol alignment with the bilayer normal in cholesterol-containing membranes is mainly determined by the ability of an analog to pack with the other cholesterol molecules in the bilayer. In a cholesterol-depleted membrane, the ability of the sterol probes to resist tilting can be more strongly affected by the linkage to the dye molecule. That is, the longer linker in phenyl-BODIPY-cholesterol causes less disturbance on the sterol orientation compared to the shorter linker in TopFluor-cholesterol. We surmise that this comes to the price of higher titling of the attached BODIPY moiety in the phenyl-linked analog. To directly test this hypothesis, we calculated the tilt angle of the BODIPY unit in both analogs, as shown in figure 7.

For the phenyl-BODIPY probe in the cholesterol-containing membrane, the angles are very widely distributed, covering almost the entire range from 0 to 180 degrees, but with two accumulations centered around 45 and 150 degrees. When going to the pure POPC membrane, the distribution becomes mostly flat across the entire range. Thus, phenyl-BODIPY has relatively uniform tilt angle distribution in both cholesterol-rich and cholesterol-depleted membranes.

In contrast, the tilt-angle distributions of TopFluor are more well-defined. In the cholesterol-containing membrane, there is a bi-modal distribution centered at 90 and 160 degrees. Here, the region around 90 degrees shows much more considerable accumulation. In the POPC membrane, simulations show that the TopFluor probe has the same two peaks, but now with the peak centered around 160 degrees having the largest accumulation.

The 2D histograms in figure 7 suggest an explanation for the cholesterol tilt angle distributions. In the cholesterol-rich membranes, the tilt angle distributions of the tagged cholesterol are quite similar (see the blue and red curves in figure 8). From the 2D histograms, we see that the BODIPY units are concentrated towards certain tilt angles (45, 150 degrees for phenyl-BODIPY and 90 degrees for TopFluor). At these angles, the tilt angle of the tagged
cholesterol is similar to that of native cholesterol.

In the cholesterol-depleted membranes (green, yellow lines of figure 8), the same situation is seen for phenyl-BODIPY-cholesterol. The BODIPY unit is mainly oriented with a tilt angle between 30 to 120 degrees, where the cholesterol tilt angle distribution is similar to that of native cholesterol. It is also apparent that more extreme tilt angles of the BODIPY unit (near either 0 or 180 degrees) forces the cholesterol towards very high tilt angles. These tilt angles have a smaller population but are responsible for the shoulder in the green curve towards high tilt angles seen in figure 8.

For TopFluor in the POPC membrane, there is a considerable accumulation of the BODIPY tilt angle around 150 degrees. From the 2D histogram in figure 7, we see clearly that this forces the connected cholesterol towards high tilt angles, which in turn is responsible for shifting the cholesterol tilt angle distribution towards higher angles (yellow lines in figure 8).

The tilt angle in figure 7 is defined such that it aligns almost perfectly with the transition dipole of the lowest singlet transition in the BODIPY unit. As a result, the tilt angle can be related to characteristics of the absorption spectrum of the BODIPY unit. For a uniform distribution of the BODIPY tilt angles, the isotropically averaged oscillator strength is valid for describing the transition, whereas sharply peaked distributions would lead to preferential absorption along a particular direction. This is then highly relevant for TopFluor, which showed a bi-modal distribution centered at 90 and 160 degrees. The peak at 90 degrees was preferentially populated in cholesterol-rich membranes, whereas the peak at 160 degrees was preferentially populated in cholesterol-depleted membranes.

Both results are in excellent agreement with experiments using two-photon excited polarimetry in giant unilamellar vesicles (GUVs); this oriented membrane system allowed us to determine the orientation of the transition dipole of TopFluor-cholesterol. In GUVs made of POPC and cholesterol, one peak centered around 10 degrees relative to the incident electric field vector was found. As this vector is shifted by 90 degrees relative to the bilayer normal, this value corresponds to around 100 degrees in figure 7, which is close to the major
peak in our calculations. In two-photon polarimetry experiments, we also found two peaks with a relative shift of 90 degrees in pure POPC GUVs. In those experiments, the value at 90 degrees relative to the bilayer normal gave a much stronger fluorescence response (see figure 2 in ref. [24]).

Overall, while both probes show rather broad distributions, TopFluor has more defined peaks compared to phenyl-BODIPY-cholesterol, precisely as expected. When going from a cholesterol-containing membrane to a pure POPC one, the angles become more uniformly distributed. Thus, bulk cholesterol in a membrane does not only order the sterol part but also restricts the mobility of the attached fluorophore, exactly as we found previously in experiments.[23]

Some care should, of course, be taken in this assessment, as it is clear from the traces that the tilt angle tends to fluctuate near particular angles and only changes over long periods. For this reason, sampling becomes an issue, and this should be taken into account when considering the distributions.

**Partial density**

The partial mass density profile of the probe-containing membranes is depicted in figure 9.

The partial mass density is shown for selected components of the membrane, including the head-groups of the lipids, the cholesterol, and the BODIPY fluorophore. When comparing the cholesterol-containing membrane to the pure POPC one, there is a noticeable increase in the membrane thickness in agreement with the data reported in table 3. We do not present the distributions of the probe-free membranes, as the mass distributions of the main membrane constituents (PC, OL, PA, CHL, water) are almost indistinguishable. In the 30% MOL cholesterol containing membranes, we can compare the placement of the tagged cholesterol to native cholesterol. Concerning the position of the tagged cholesterols (red lines), the position of maximum density coincides, for both the TopFluor and phenyl-BODIPY tagged probes, with the position of native cholesterol. For the pure POPC membrane, there is no
Figure 9: Partial density of selected components of the studied membrane-probe systems is shown. The mass density is split into contributions from TopFluor or BODIPY in phenyl-BODIPY (BODIPY), from the phenyl group in phenyl-BODIPY, from the tagged cholesterol (BODIPY-CHL) and from regular cholesterol (CHL), the head groups (PC) and water. For clarity, the densities of the probes and tagged cholesterol have been scaled up by a factor of 64.
native cholesterol to compare to, but the two tagged cholesterol distributions are again near each other.

In short, there is no significant difference induced by including the tagged cholesterols in terms of the mass distributions of the major components of the membrane, and in terms of the mass distribution of the tagged cholesterol itself. The major difference between the two probes is instead to be found where the BODIPY unit resides in the membrane. With the TopFluor probe in a cholesterol membrane, the BODIPY unit is found almost exactly in the middle of the membrane. The mass distribution is tight, with a slight outward-facing tail. In a POPC membrane, the central peak of the mass density of TopFluor diminishes, and the distribution becomes broader and shifts outwards along the direction in which the tagged cholesterol resides. In both cholesterol and POPC, the phenyl-BODIPY mass distribution is wider than the TopFluor one. This is in part due to the large size of the probe, but perhaps more importantly due to the increased flexibility of the fluorophoric unit due to the phenyl linker. This is in line with the broad distributions of the BODIPY unit tilt angle of phenyl-BODIPY seen in figure 7.

Phenyl-BODIPY Rotor function

Instrumental for the function of phenyl-BODIPY-cholesterol as a molecular rotor is the possibility of rotation between the BODIPY unit and the phenyl ring. This rotation is supposed to affect the quantum yield and excited-state lifetime of the probe and thereby report about local membrane viscosity. For molecular rotors, the viscosity can experimentally be related to the fluorescence quantum yield via the Förster-Hoffman relation

\[ \Phi_f = z\eta^\alpha, \]

where \( z \) and \( \alpha \) are constants. By writing the fluorescence quantum yield in terms of the radiative and non-radiative decay constants.
the fluorescence lifetime, $\tau_f$, can therefore be related to the viscosity as

$$\tau_f = \frac{z\eta^\alpha}{k_r}.$$  

(5)

For more detailed discussions on the topic, we refer the reader to recent reviews.\textsuperscript{75–78}

Under the assumption that intramolecular rotation of the dye is the main contributor to the non-radiative de-excitation pathways, the rotational dynamics of the phenyl-BODIPY dye will, therefore, influence its fluorescence lifetime.

The relevant rotational dynamics occur between the BODIPY unit and the phenyl ring. We investigate these dynamics by computing the dihedral angle connecting the two groups (between atoms CB19-CB20-CB21-CB22, see figure [? for the definition) and analyzing its temporal autocorrelation function (ACF) from the MD trajectories. From the distributions of the dihedral angle, shown in figure [10], we observe a distribution with two large peaks centered at 50 and 130 degrees. Transitions between these two minima are evidently possible, with the transition going through a state with the main BODIPY ring system orthogonal to the phenyl ring. Considering traces of the dihedral angle, we observe the existence of both many short-lived transitions in rapid succession, but also several longer-lived states with life-times on the order of nanoseconds.

The ACF clearly decays faster in the pure POPC membrane than in the cholesterol-containing membrane, indicating that relaxation of phenyl-BODIPY-cholesterol is slowed down in membranes containing cholesterol, which underlines the suitability of this probe as a molecular rotor in cholesterol-containing membranes.

To quantify decay rates, we attempted fits of the decay of the ACF using both single and double exponentials but were not able to obtain good agreement with the measured data (not shown). We were, however, able to obtain excellent fits through fitting to a stretched
exponential. This allowed us to quantitatively compare the decay of the ACF in membranes made of POPC or POPC + cholesterol. The ACF and the stretched exponential fits are shown in figure 10, while the fitting parameters are given in table 4. We additionally attempted fitting the ACF with a triple exponential fitting function which gave comparably good regression results and confirmed that the dynamics of the probe is significantly slowed down in cholesterol-containing membranes (see supporting info).

A stretched exponential is described by

\[ C(\tau) = \exp \left( - \left( \frac{\tau}{\tau_0} \right)^\beta \right). \] (6)

The stretched exponential is an extension of a classical exponential decay, in which a time-dependent rate coefficient replaces the rate constant in the underlying differential equation. This allows for describing polydisperse systems with a multitude of relaxation times, i.e., a distribution of rate constants. Kohlrausch has first introduced the stretched exponential in 1854, and this has since been used to describe non-exponential relaxation processes, e.g., in luminescence decays. The stretched exponential function describes faster than exponential decays for short times compared to the time constant \( \tau_0 \) and power-law behavior for long times, suggesting that the rotation dynamics of phenyl-BODIPY in the complex environment of a lipid membrane involves multiple time scales. The ACF probes the transition between the two major dihedral angles, as can be seen by the fact that when removing all but the large-scale motions (i.e., rounding to the nearest major dihedral angle), the resulting ACF is almost identical to the original one (see the supporting information).

In experiments, the rotor function of phenyl-BODIPY-cholesterol is assessed by fluorescence lifetime measurements, directly reflecting the dynamics of the molecular rotor in any given environment. Several studies report multi-exponential fluorescence decays for other phenyl-BODIPY based rotor molecules in lipid bilayers and live cells. Such decay-processes are inherently linked to the heterogeneous distribution of the probes in membranes together with the complex dynamics of the lipid bilayer. We observe multi-exponential decay of the
ACF (see figure 10) and multi-modal tilt angle distributions of the fluorophore unit (see figure 7) in lipid bilayers, suggesting that the dynamics and orientation of this cholesterol probe in membranes are indeed heterogeneous.

The concept of viscosity, when applied to membranes, has to be adapted to account for spatially and temporally varying viscous drag in different regions of the lipid bilayer as well as to dynamic fluctuations of the membrane itself. In fact, the decay of density and thickness fluctuations as well as of thermally excited undulations of lipid bilayers has been shown to be well-described by a stretched exponential decay model. Interestingly, the fitted time constant and stretching parameter varies as a function of the wave vector in the correlation analysis for a simple phospholipid membrane, as shown in ref. A stretched exponential fit to the data is suitable for this situation, as it accounts for this heterogeneity in a statistical sense.

For a quantitative comparison of the dynamics of phenyl-BODIPY-cholesterol in the two membrane systems, we calculated the time-averaged, $\bar{\tau}$, and ensemble-averaged time constants, $\langle \tau \rangle$, of the stretched exponential decay of the ACF defined by

$$\bar{\tau} = \frac{\int_0^\infty \tau C (\tau) \, d\tau}{\int_0^\infty C (\tau) \, d\tau}$$

(7)

$$\langle \tau \rangle = \int_0^\infty C (\tau) \, d\tau.$$  

(8)

Using the parameters shown in table we find by numerical integration of eq. (7) a time-averaged time-constant of 7.49 ns in the POPC membrane, while the POPC membrane with cholesterol shows $\bar{\tau} = 48.46$ ns. Meanwhile, the ensemble-averaged time constant, $\langle \tau \rangle$, is 1.37 ns and 7.33 ns for the pure POPC membrane and the POPC membrane with cholesterol, respectively. Thus, the rotation dynamics of phenyl-BODIPY-cholesterol is slowed down by a factor of 5–6 in cholesterol-containing membranes compared to cholesterol-free bilayers. This prediction can be tested by fitting experimental fluorescence lifetime decays with a
stretched exponential model in future experiments. Fitting a stretched exponential has been shown to be superior to multi-exponential fitting in describing fluorescence kinetics, such as lifetime and anisotropies decays or photobleaching, and can be easily implemented on a pixel-by-pixel basis for imaging applications as well.85–87

The stretched exponential decay of the autocorrelation function of the phenyl-BODIPY dihedral angle in membranes made of POPC or POPC + cholesterol reflects the complex relaxation dynamics of the probe in the heterogeneous membrane environment. We suggest that our results should be compared to stretched exponential fits to the fluorescence lifetime and eventually anisotropy decay in future studies, thereby allowing for a direct comparison of experiment and theory.

Table 4: The autocorrelation function of the dihedral angle was fitted to a stretched exponential, see eq. 6. Parameters relating to the quality of the fit (χ², R²) are also reported. The fit was made with the non-linear regression tool from xmgrace.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>τ₀ (ns)</th>
<th>β</th>
<th>χ²</th>
<th>R²</th>
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</thead>
<tbody>
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<td>0.399</td>
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<td>0.9972</td>
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<tr>
<td>CHL</td>
<td>1.826</td>
<td>0.376</td>
<td>0.06554</td>
<td>0.9980</td>
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</tbody>
</table>
Figure 10: Autocorrelation function of the phenyl-BODIPY dihedral angle (top panel) and distributions of the dihedral angle (bottom panel) in POPC or 30% MOL cholesterol membranes. A fit to a stretched exponential is shown as dashed lines. The inset in the lower panel shows a typical trace of the dihedral angle at an arbitrarily selected point along the trajectory of phenyl-BODIPY in the POPC membrane.
Conclusion

Molecular rotors attached to lipid molecules are unique tools for the investigation of local membrane packing and viscosity. In this study, we have carried out a detailed computational analysis of a recently developed phenyl-BODIPY rotor attached to the side chain of cholesterol and have compared its properties to that of the widely used TopFluor-cholesterol. Having established an improved parametrization of the dye for molecular simulations using force-matching, we perform a direct comparison of phenyl-BODIPY-cholesterol with the commercially available BODIPY-based cholesterol analog TopFluor-cholesterol. The latter probe has been used in many biophysical and cell biological studies. Phenyl-BODIPY-cholesterol differs from TopFluor-cholesterol only by having a phenyl group between cholesterol's aliphatic side chain and the BODIPY moiety (and by the presence of the methyl group substituents). We demonstrate that both analogs do not perturb the membrane bilayer significantly as long as they are used at very low concentrations. The orientation distribution of the long molecular axis of the BODIPY moiety coincides with its transition dipole moment, and we show that this distribution is bimodal for TopFluor-cholesterol in a cholesterol-dependent manner, which is in excellent agreement with experiments. For phenyl-BODIPY-cholesterol, this bimodal distribution is less pronounced, suggesting that the extended linker via the phenyl group creates more structural flexibility in the membrane. Attaching the dye increases the range of tilt angles of the linked cholesterol compared to native cholesterol, and this effect was more pronounced for TopFluor-cholesterol compared to phenyl-BODIPY-cholesterol. An increased sterol tilt angle suggests that the ability of BODIPY-linked cholesterol analogs to align with the fatty acyl chains of POPC is reduced compared to that of pure cholesterol, and this effect is augmented in bilayers containing cholesterol. This is in excellent agreement with recent NMR studies on TopFluor-cholesterol in model membranes. However, as TopFluor-cholesterol and phenyl-BODIPY-cholesterol would be used at low concentrations of less than 1 mole percent in experiments, their inability to condense the bilayer should be less of a concern. By analyzing the ACF of the phenyl-
BODIPY rotation angle, we find that the rotation dynamics involves multiple time-scales and that a high-concentration cholesterol environment leads to a slower rotation. This establishes phenyl-BODIPY-cholesterol as a promising candidate for sensing cholesterol induced membrane microviscosity in living cells in future experimental studies.

Acknowledgement

Computations/simulations for the work described herein were supported by the DeIC National HPC Centre, SDU. We acknowledge the Danish Council for Independent Research for financial support (Grant ID: DFF–7014-00050B) and the H2020-MSCA-ITN-2017 COSINE Training network for COmputational Spectroscopy In Natural sciences and Engineering (Project ID: 765739) for financial support.

Supporting Information Available

Calculations of the transition dipole moment of the lowest singlet excited state of phenyl-BODIPY. Triple-exponential fits of the ACFs of the phenyl-BODIPY rotor dihedral angle along with additional ACFs on modified time-series of the dihedral angle. Optimized force-field parameters of linked/unlinked phenyl-BODIPY and TopFluor molecules.

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