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Rational Design of Nile Red Analogs for Sensing in Membranes

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Abstract

Development of next-generation fluorescent probes is a key element in the quest for a greater understanding of complex biological environments (e.g., membranes) by bioimaging. Such fluorescence-based techniques rely on specialized small molecules that possess excellent fluorescent properties but also do not perturb the native biological environment in which they reside. Herein we present a theoretical/computational strategy for the design of novel optical probes for sensing in membranes based on the parent chromophore Nile Red. Using a combination of time-dependent density functional theory (TD-DFT) and molecular dynamics (MD), we have studied the optical properties and accommodation in a model membrane of Nile Red and eight analogs. Special attention has been given to the design of probes with improved solvatochromism and two-photon absorption (2PA) without altering the membrane properties. Of the eight studied analogs, two probes were found to possess attractive probe features and are hence suggested to be taken forward to chemical synthesis and experimental exploration.

Introduction

Computational chemistry has today become a key component in rational design of novel molecules with tailored properties, not least in the development of probes for sensing in complex environments. From a computational point of view, this is a challenging task as the environment plays a decisive role and must to be included in the design process, thereby often requiring consideration of sizeable molecular systems. In this paper, we demonstrate a computational procedure that can be efficiently used to consider rational design processes of probe molecules with the aim of generating novel molecules possessing increased sensitivity to a given environment; in this specific case, a membrane. Our starting point for the search of new probe molecules is the lipophilic fluorescent dye Nile Red. The optical properties, and especially the emission energy, of Nile Red are already quite sensitive to the polarity
of the environment.\textsuperscript{1,2} This sensitivity appears to originate from the relatively large gap between the ground and excited-state dipole moments, thus leading to a differential stabilization of the two states upon introducing Nile Red into polar environments. The excited state is associated with an intramolecular charge transfer (ICT) that occurs between the donor (diethylamino) and the acceptor (ketone) parts of the molecule. The nature of the ICT excited-state structure is still debated, yet it is likely that the conformation of the diethylamino group plays an important role,\textsuperscript{3} and that the twisted conformation of Nile Red can be attributed to the high sensitivity to the environment and the large Stokes shift.\textsuperscript{3,4} Due to its high solubility in lipids, Nile Red has found wide application within bioimaging of lipid membranes.\textsuperscript{5} However, contemporary bioimaging techniques are limited by the lack of suitable probes that feature the requisite characteristics for efficient usage. For instance, many two-photon excitation microscopy techniques use standard one-photon probes.\textsuperscript{6,7} Such probes are not optimized for two-photon applications resulting in very low intensity, thereby limiting their use. Certainly, there is an urgent need for designing more efficient probes with enhanced two-photon absorption (2PA) properties that can be used with high specificity.

In this study, a design strategy was launched to find practical ways to improve the 2PA intensities and optical properties that dictate the polarity sensitivity (e.g., the change in dipole moment upon excitation). In addition, the excitation energy must be taken into consideration as it should be as low as possible to diminish phototoxicity. Finally, from a structural point of view, the probes should interfere as little as possible with the membrane structure and functionality.

It is known that molecules with extended $\pi$ systems such as Nile Red that feature end-capped donor and acceptor groups make up potent push-pull systems.\textsuperscript{8} The interaction between the donor and the acceptor moieties leads to intramolecular charge transfer, causing the molecule to become more polarized. This polarization usually leads to a significant change in dipole moment upon excitation. This feature can translate into a higher sensitivity to the
environment as well as a non-linear property enhancement to which the 2PA cross-section is directly related. In this study, we intend to increase the 2PA intensity by augmenting the push-pull system of the parent Nile Red molecule. In order to select a rational subset of potential analogs, we performed an initial screening of numerous push/pull substituents at all vacant aromatic positions of Nile Red using a rather low level of theory to compute one- and two-photon intensities. We noticed from this initial screening that the installation of either push or pull substituents in the two terminal rings of Nile Red significantly increases the 2PA cross-section. From this initial screening, and with an emphasis on synthetic feasibility (given the experimental vision of this study), we selected eight promising Nile Red analogs for further analysis.

In this paper, we first investigate the spectroscopic properties of the selected analogs in gas-phase with a special focus on two-photon absorption and change in dipole moments upon excitation. Next, we address the behavior of the probes inside the lipid bilayer based on molecular dynamics. We have focused on two commonly used structural parameters, namely the membrane thickness and the area per lipid. Finally, calculations of the optical properties of the probes in a model membrane are carried out within the scheme of polarizable embedding.

**Computational Details**

**Preliminary screening**

In order to select promising candidates for further detailed studies, an initial screening procedure was carried out. Using the RDKit python package, singly substituted analogs of the Nile Red molecule were created by generating all possible replacements of an aromatic hydrogen with a new substituent. The substituents included "pull"-type (−C≡N, −NO₂, −CF₃, −SO₃⁻, −F, −CHO, −COCH₂CH₃, −COOH) and "push"-type (−NEt₂, −NHEt, −NH₂, −OH, −O⁻, −OCH₃, −NHCOrt, −OCOCH₂CH₃, −CH₃, −CH₂CH₃) groups. Nile
Red contains eight aromatic hydrogens, leading to a total of $8 \times 18 = 144$ compounds. The python script used to generate these compounds is given in the Supporting Information.

The geometry of each of the newly built molecules was optimized in a three-stage protocol. First, an initial rough optimization with the MMFF94$^{9,10}$ force-field was done. Next, an optimization using the semiempirical HF-3c$^{11}$ method in Orca$^{12,13}$ was performed, followed by a final optimization at a B3LYP$^{14}$-D3$^{15}$/6-31G* level of theory.

One- and two-photon properties of the new analogs were then computed in Dalton$^{16}$ using the range-separated CAM-B3LYP$^{17}$ functional with the 6-31G* basis set. For one-photon properties, five states were computed, whereas only the lowest excited state was considered for two-photon properties.

**Electronic structure calculations**

All ground-state gas-phase geometry optimizations have been carried out using density functional theory (DFT) with the B3LYP$^{14}$ exchange correlation functional. The excitation energies and one-photon absorption (1PA), optimizations of the lowest excited state, emission energies and two-photon absorption cross sections ($\sigma^{2PA}$) have been calculated using the CAM-B3LYP$^{17}$ functional within the time-dependent DFT (TD-DFT) formalism. The 6-311++G**basis set$^{18}$ was employed. All calculations have been carried out using Gaussian09$^{19}$ except the 2PA cross sections which were computed using Dalton.$^{16}$

**MD simulations**

The MD simulations have been performed using the Amber14$^{20}$ package. An initial lipid bilayer structure has been generated using the CHARMM membrane builder GUI.$^{21}$ The overall membrane consists of 64 POPC lipids in each leaflet, along with 40 water molecules per lipid, giving a total of 5120 TIP3P$^{22}$ water molecules. Ions (0.15M KCl) were added through a Monte-Carlo placement method. The Lipid14$^{23}$ force field was used to describe the POPC lipids, water molecules were described with the TIP3P model,$^{22}$ while parameters
developed by Cheatham et al. were used for the ions.\textsuperscript{24} The assembled systems were converted to Lipid14 PDB format by means of the charm-lipid2amber.py\textsuperscript{20} script. Probes were inserted into the membrane of this initial system by replacing 10 of the 128 total lipids with an equal number of probes (5 for each leaflet). This corresponds to a concentration of 0.084 probes/lipid. For one of the probes, a smaller concentration was realized by only replacing a single lipid in each leaflet.

Parameters, topologies, and partial atomic charges of the probes were derived using Antechamber.\textsuperscript{25} Charges were derived using the RESP\textsuperscript{26} procedure (B3LYP/6-311++G**), while the remaining parameters were assigned using the GAFF\textsuperscript{27} force field. The initial size of the periodic box was assigned from the extent of the water molecules present at the borders of the box.

The assembled systems were then equilibrated in a multi-stage procedure. First, minimization was used to relax the initial structures using 10000 cycles, of which the first 5000 were based on the steepest descent method while the remaining steps were performed with a conjugate gradient minimizer. The volume was kept constant throughout the minimization. The systems were heated slowly to the production temperature of 303 K in two stages using the Langevin thermostat.\textsuperscript{28} In the first stage, the systems were heated from 0 K to 100 K in 2500 steps at constant volume. In the second stage, the systems were taken from 100 K to the 303 K across 50000 steps. An anisotropic Berendsen weak-coupling barostat\textsuperscript{29} was used to control the pressure towards 1 bar. A time-step of 2 fs was used for these stages, and all the following. Long-range electrostatics were treated using the Particle Mesh Ewald method with a cut-off of 10 Å. In both heating stages, the lipids were restrained towards their initial positions (force constant 10 kcal mol\textsuperscript{-1} Å\textsuperscript{-2}). Bonds involving hydrogens were constrained using the SHAKE\textsuperscript{30} method. Next, an initial equilibration of the systems was carried out in the NPT ensemble (without restraints) for 5 ns.

Finally, production simulations were carried out for 200 ns. Of the total simulation time, the first 80 ns were discarded to allow complete equilibration of the membrane structure and
convergence of the area per lipid. From the remaining 120 ns of the production trajectory, frames were saved every 10 ps for the following structural analyses. For the embedding calculations, snapshots were extracted from the production trajectory every 2 ns for a total of 61 snapshots per probe.

**Introduction of environment effects**

In order to include environmental effects into the optical property calculations, the polarizable embedding\(^{31,32}\) (PE) method was used. In these calculations, one of the probe molecules was described by TD-DFT, while the remaining molecules (lipids, water, ions, and the probes) were described classically by means of an embedding potential. The parameters in the embedding potential were assigned using the PyFraME python package.\(^{33}\) For water,\(^{34}\) ions and lipids,\(^{35}\) the built-in isotropic parameters were used. For the other probe molecules, structure-specific parameters were derived based on the LoProp\(^{36}\) method. These potentials consist of static multipoles (up to and including quadrupoles) and dipole-dipole polarizabilities distributed to atomic centers. All of these have been calculated with the B3LYP functional using the recontracted ANO-type loprop-6-31+G* basis set. The optical properties of the selected probe were calculated with the fully long-range corrected CAM-B3LYP functional \((\mu = 0.33, \alpha = 0.19, \beta = 0.81)\) with a 6-31G* basis set. All PE calculations were carried out using the Dalton\(^{16}\) program.

**Results and discussion**

**Preliminary screening**

Figure 1 presents a summary of the computed one- and two-photon data for the screening of singly substituted Nile Red analogs. Among the five lowest excited states of Nile Red, only the lowest one is bright for a one-photon process. For the single-substituted Nile Red analogs, the excitation energies and oscillator strengths are distributed according to an
overall pyramid-like shape with the Nile Red molecule towards the top of the pyramid, i.e., the oscillator strengths of the new analogs tend to be largest when the excitation energy is close to that of the lowest-lying state in Nile Red. Most of the new compounds have worse optical properties than Nile Red, but a few compounds show an increased oscillator strength. The highest oscillator strength from the singly substituted set is 1.02, compared to 0.85 for Nile Red. Thus, there are no significant improvements to be gained in terms of one-photon properties.

A similar distribution is seen for the two-photon strengths, but now with the Nile Red molecule being more towards the middle of the pyramid. Whereas Nile Red has a predicted 2PA cross section ($\sigma^{2PA}$) of 52.2 GM, the top singly substituted candidate shows a $\sigma^{2PA}$ of 89.6 GM. Some clear trends are evident from the structures of the top candidates, namely that the best performers have introduced a pull-group in the lower-right ring or a push group on the left-most ring. This arrangement aligns nicely with the existing pull/push framework of the unsubstituted Nile Red molecule. For the singly substituted Nile Red molecules, both push and pull groups appear in the top set, indicating that the introduction of either kind of group can be beneficial.

Based on the initial screening procedure, eight analogs of Nile Red (NR) have been designed by introducing specific electron-withdrawing (pull) or -donating (push) substituents to the benzophenoxazine scaffold (see Figure 2) to examine how substitution alters the spectroscopic properties of NR. This enables us to collect information about the optimal probe design for achieving superior sensitivity to membrane environments and ideal absorption properties. The gas-phase calculations allow for studying all the analogs in a reasonable compromise between accuracy and cost. Based on these gas-phase calculations, promising molecules can be subjected to more detailed investigations that include the influence of the environment: first, in terms of how the probes modify membrane properties, and second, in how the membrane environment modifies the optical properties of the probe.
Figure 1: Screening results of one- and two-photon properties of singly substituted Nile Red analogs. Panel A shows the distribution of excitation energies and associated oscillator strengths (length gauge) of the five lowest excited states. Panel B shows the distribution of two-photon absorption strengths for the lowest excited state. The five highest-ranked compounds in terms of 2PA are shown in panel C.
Electronic structure calculations

The lowest-lying electronic excitation energies, as well as associated properties for all analogs of Nile Red (see Figure 2) are given in Table 1. Except for analog 4, the absorption energy of the analogs are red-shifted (by 3 to 25 nm) with respect to NR. Likewise, the emission energy is generally red-shifted compared to NR (again, except for 4). In terms of increasing the Stokes shift, the largest improvements are observed for analog 8, with the remaining analogs being almost indistinguishable from the parent Nile Red molecule. Unfortunately, the increase in the stokes shift for analog 8 is accompanied by a significant decrease in the OPA intensity (decrease in the oscillator strength from 0.86 to 0.63).

Lipids bilayers are structurally and chemically diverse, so probes that can sense such environmental differences, and especially the amount of water in the membrane, are of great value. A significant difference in dipole moment upon excitation can be used by the probe to respond to such differences in the local dielectric environment. The consequent stabilization
Figure 3: (a) The most stable conformation of NR in gas-phase, and (b) resonance structures indicating the charge transfer mechanism of NR.

or destabilization of the excited state results in either red-shift or blue-shift of the absorption and emission wavelength. All the analogs have an increase of dipole moment when going from the ground to excited state. Consequently, more polar environments will stabilize the excited state and lead to red-shifted absorption and emission. Two of the probes are special in this regard, namely 3 and 8, which have much larger changes in the excited state dipole moment (4.35 and 5.06 D) than the parent Nile Red molecule (3.78 D) and the remaining probes. The improved performance of 3 and 8 can be rationalized in terms of enhanced charge displacement from the donor to the acceptor part of the molecule, leading to larger changes in the dipole moment upon excitations. This enhanced change in dipole moment upon excitation for 3 and 8 can be understood based on the locally excited (LE) and twisted intramolecular charge transfer (TICT) structure hypothesis. For more details see SI and references herein.

The calculated 2PA cross-sections $\sigma^{2PA}$ for the lowest-lying transitions are given in Table 1. Only one of the analogs, 3, has a significantly higher calculated $\sigma^{2PA}$ (79 GM) than Nile Red (62 GM), whereas another, 4, shows a much smaller cross-section (50 GM). For the remaining analogs, the calculated cross sections are similar to the parent Nile Red molecule. A two-state model for the 2PA can partially explain this observation. In such a model,
Table 1: Spectroscopic details computed for the analogs 1-8 as well as for Nile Red (NR) in gas-phase. The excitation and emission energies $\Delta E$ and Stokes shifts are in eV and in nm (in parentheses). Also reported for each transition are the oscillator strength $f$, the two photon absorption (2PA) cross section $\sigma^{2PA}$ quoted in GM ($10^{-50}$ cm$^4$ photon$^{-1}$ s), a Lorentzian broadening with a full-width at half maximum of 0.1 eV is assumed), the magnitude of the transition dipole moment $\mu_{0f}$ in Debye, and the change in the dipole moments upon excitation $\Delta\mu_{0f}$.

| Probe | $\Delta E$ ($\lambda$) | $f$ | $|\mu_{0f}|$ | $\Delta\mu_{0f}$ | $\sigma^{2PA}$ | $\Delta E$ ($\lambda$) | $f$ | $|\mu_{0f}|$ | $\Delta\mu_{0f}$ | $\Delta\Delta E$ ($\Delta\lambda$) |
|-------|------------------------|-----|-------------|----------------|-------------|------------------------|-----|-------------|----------------|------------------|
| NR    | 2.97 (417)             | 0.86| 8.74        | 3.92           | 62.5        | 2.64 (469)             | 0.83| 9.08        | -0.33          | 52               |
| 1     | 2.92 (424)             | 0.87| 8.87        | 4.03           | 69.9        | 2.61 (475)             | 0.83| 9.15        | -0.31          | 51               |
| 2     | 2.91 (426)             | 0.88| 8.95        | 3.98           | 69.5        | 2.62 (473)             | 0.85| 9.23        | -0.29          | 47               |
| 3     | 2.94 (421)             | 0.85| 8.74        | 4.56           | 78.8        | 2.62 (473)             | 0.85| 9.25        | -0.32          | 52               |
| 4     | 3.00 (413)             | 0.85| 8.66        | 3.61           | 50.7        | 2.65 (467)             | 0.82| 9.03        | -0.35          | 54               |
| 5     | 2.80 (442)             | 0.80| 8.68        | 3.93           | 67.7        | 2.50 (496)             | 0.76| 8.94        | -0.30          | 54               |
| 6     | 2.95 (420)             | 0.88| 8.86        | 4.03           | 75.6        | 2.65 (469)             | 0.85| 9.22        | -0.31          | 49               |
| 7     | 2.95 (421)             | 0.85| 8.71        | 3.87           | 77.0        | 2.62 (473)             | 0.80| 8.97        | -0.32          | 52               |
| 8     | 2.84 (437)             | 0.63| 7.66        | 5.29           | 67.4        | 2.38 (521)             | 0.45| 7.09        | -0.46          | 85               |

The cross-section is determined by the difference dipole moment $\Delta\mu_{0f}$, the transition dipole moment $\mu_{0f}$, and the angle $\theta$ between these dipole moments

$$\sigma^{2PA} = \frac{16\pi^3\alpha a_0^5}{c} \frac{1}{\pi\Gamma} |\mu_{0f}|^2|\Delta\mu_{0f}|^2(2\cos^2(\theta) + 1),$$  

where $\alpha$ is the fine structure constant, $a_0$ is the Bohr radius, $\Gamma$ is a broadening parameter, and $c$ is the speed of light. As seen from Table 1, the transition dipole moments are of similar magnitude, while the variation in the difference dipole moments is much more significant. Thus, the latter is expected to have a more profound impact on the $\sigma^{2PA}$, and this rationalizes the large cross-section of 3. Meanwhile, the largest difference dipole moment occurs in 8, but uniquely, this probe also sees a significant decrease in the transition dipole moment. As a result, the two-photon cross section is only slightly larger than that of the parent Nile Red molecule.
MD simulations

MD simulations have been performed for the pure POPC lipid bilayer and for POPC, including each of the probes in Figure 2. Each system consists of a total of 10 probes randomly placed within the lipid bilayer. These simulations aim to estimate the perturbation that a probe induces in the membrane. This is evaluated as a direct comparison with the computed properties of a pure membrane as well as with the membrane containing NR, which is, based on experimental procedures, a very well working fluorescent dye for bioimaging applications.\textsuperscript{37} The bilayer thickness and the area per lipid have been used as evaluating parameters to assess how much each probe perturbs the membrane. Additionally, the order parameters, the density along the lipid bilayer, and the tilt angle distribution (for the probes considered) have been calculated. The bilayer thickness has been defined as the average distance between the center of mass of the phosphorus atoms of the phosphatidylcholine groups on the upper leaflet and the same on the bottom leaflet.

Table 2: Membrane parameters for a pure POPC membrane and for membranes incorporating Nile Red analogs. The area per lipid is reported as Å\textsuperscript{2}/lipid while the membrane thickness is given in Å. Moreover, for each average, the block error is reported. All the reported data has been calculated with MDanalysis and analyzed with gmx analyze tool present in Gromacs.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Membrane thickness (Å) average</th>
<th>Membrane thickness (Å) block error</th>
<th>Area/lipid (Å\textsuperscript{2}/lipid) average</th>
<th>Area/lipid (Å\textsuperscript{2}/lipid) block error</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR</td>
<td>40.288</td>
<td>0.007</td>
<td>62.493</td>
<td>0.013</td>
</tr>
<tr>
<td>1</td>
<td>40.587</td>
<td>0.005</td>
<td>61.999</td>
<td>0.009</td>
</tr>
<tr>
<td>2</td>
<td>40.258</td>
<td>0.005</td>
<td>62.770</td>
<td>0.009</td>
</tr>
<tr>
<td>3</td>
<td>39.587</td>
<td>0.007</td>
<td>64.291</td>
<td>0.014</td>
</tr>
<tr>
<td>4</td>
<td>40.485</td>
<td>0.007</td>
<td>61.920</td>
<td>0.012</td>
</tr>
<tr>
<td>5</td>
<td>40.302</td>
<td>0.006</td>
<td>62.787</td>
<td>0.012</td>
</tr>
<tr>
<td>6</td>
<td>39.711</td>
<td>0.006</td>
<td>63.585</td>
<td>0.011</td>
</tr>
<tr>
<td>7</td>
<td>40.681</td>
<td>0.005</td>
<td>61.685</td>
<td>0.008</td>
</tr>
<tr>
<td>8(ten)</td>
<td>40.038</td>
<td>0.005</td>
<td>63.886</td>
<td>0.009</td>
</tr>
<tr>
<td>8(two)</td>
<td>39.451</td>
<td>0.005</td>
<td>62.658</td>
<td>0.010</td>
</tr>
<tr>
<td>pure</td>
<td>39.462</td>
<td>0.005</td>
<td>62.127</td>
<td>0.009</td>
</tr>
</tbody>
</table>

The membrane thickness computed for the pure POPC bilayer agrees with the experimental
value (39.8 Å at 20°C).³⁸,³⁹ For the remaining membranes, an increase in the membrane thickness would be expected when external probes are embedded.⁴⁰ Indeed, in all cases, this is observed. However, the increases are in absolute terms rather modest, with the largest increase in the membrane thickness, observed for analog 7, being just 1.2 Å.

The area per lipid has been calculated as the total membrane area divided by the number of lipids in one leaflet, i.e., 64 for the pure bilayer and 59 for all the other analogs. As with the membrane thickness, only minor changes are observed upon incorporating the analogs into the POPC membrane. The largest differences occurs with 3, 6 and 8, for which the area per lipid decreases by 2.2, 1.5, and 1.8 Å², respectively. The remaining probes generally see slight increases, except for 1, 4, and 7, where the area per lipid is reduced with respect to the pure POPC membrane. Thus, these probes are inducing slight condensing effects in the bilayer. A decrease in the area per lipid is commonly observed for small and hydrophobic probes.⁴¹–⁴³ Also, in this case, the area per lipid for the pure bilayer is in accordance with the experimental one (62.7 Å² at 20°C).³⁸,³⁹ Nile Red is among the probes that lead to the smallest perturbations of the area per lipid. Nevertheless, it is not too different from the analogs, and variations of the order observed for the analogs can, therefore, be considered acceptable for applications.

Concerning the deuterium order parameter, $S_{CD}$, the introduction of the different probes induce a significant change in $S_{CD}$ compared to the pure membrane for both tails (see Figure 4). All analogs, NR included, systematically increase the order parameters. In the sn-1 tail, the smallest effects are seen with 3, 6, and 8, which all show order parameters very similar to that of the pure POPC membrane. The remaining analogs are clustered together and increase the order parameters more substantially (for example, the order parameter of C6 increases on average from 0.22 to 0.24). In the sn-2 tail, there is not such a clear splitting into two groups, but again, analogs 3, 6, and 8 are among the least perturbing.

Figure 5 shows where the density of the probes along the $z$-axis of the membrane. The probes
are distributed into two layers corresponding to the upper and lower leaflet of the membrane.

The mass distributions are bimodal, centered at about ±1 nm into the membrane. These
positions correspond to the middle of a leaflet. Most analogs show non-zero density in the
middle of the membrane, indicating that transfers of a probe from one leaflet to another
are possible on the time-scales of the simulations. The upper and lower leaflets typically
have equal populations, except for analogs 2 and 6, which show considerable accumulation
in the lower and upper leaflets, respectively. A visualization of the MD trajectory (see
Figure 6) clearly shows that this accumulation happens as a result of the creation of stable
agglomerates. This accumulation may introduce complications for experimental use of these
two probes.

Figure 4: Deuterium order parameters for the sn-1 (a) and sn-2 (b) segments of POPC in
the pure and in the embedded bilayer. The numbering of the POPC segments is reported
in SI.
Figure 5: Density distribution of the probes across the bilayer. The density profiles are averaged over the 120 ns of the MD.

Figure 6: MD snapshot of analog 6 embedded in the membrane lipid bilayer.

Figure 7 shows the tilt angle distribution of each analog with respect to the bilayer normal. The tilt angle is defined as the angle formed by the intramolecular vector $C_{10}$ and $C_{4a}$ (see...
Figure 2 for numbering) and the membrane normal. In order to resolve this into upper and lower leaflets, the tilt angle is resolved also along the $z$-position in the membrane, giving rise to a two-dimensional (tilt-angle, $z$-position) frequency plot. For Nile Red, and the majority of the analogs, there is a preferential distribution of the tilt angle at around 160/20 degrees for the upper/lower leaflets, with a broad background from 0–180 degrees. The analogs 2 and 6 deviate from this pattern due to the previously discussed formation of agglomerates and show considerable accumulation into a single leaflet. Since the dimers formed by these dimers tend to be in anti-parallel arrangements (see Figure 6), we see accumulation in the tilt angle at 20/160 degrees, but in the same leaflet.

**Low concentration**

Additional MD simulation has been performed for 8 to investigate the effect of lower probe concentrations. This analog was selected since it represents, together with analog 3, one of the most promising probes as seen from the analysis of the electronic structure properties. As expected, using a lower concentration of the probe reduces the perturbation in both membrane thickness and the area per lipid (see 8(ten) and 8(two) in Table 2). Inspecting the order parameters (see Figure 4), for analog 8(two) we observe minimal variation resulting in an almost perfect overlap with the properties of the pure membrane. However, the order induced by 8(ten) and 8(two) is quite similar even though a bit more extended for 8(ten) as could be expected when the concentration is increased. Finally, it is observed that analog 8(two) distributes equally in the two leaflets, far away from the central part of the bilayer (see Figure 5). The tilt angle distribution, see Figure 7, is mainly localized around the interval between 60 and 120 degrees. Both of these observations are in perfect agreement with what has already been observed for 8(ten).
Figure 7: Tilt angle density distribution for all the analogs within the lipid bilayer. The number density of the probes inside the membrane is represented by the 'hot' colour map on the right side of each graph and is normalized to 100.

**Environment**

So far, we have considered optical properties of the suggested probes based on calculations made on the probes in isolation. It is, however, well known that the effect of an external environment can influence optical properties significantly. Thus, we here consider the calculation of key optical properties, including explicitly the presence of the membrane. For this, 61 snapshots have been extracted from the last 120 ns of the MD simulations of the probes, and 1PA and the 2PA properties have been calculated using PE based on these snapshots.
Due to the computational cost of calculating higher-order response properties, we have for the 2PA only considered analogs 3 and 8. Averages of these optical properties are reported in Table 3 and are compared with the gas-phase properties calculated with the same level of theory (distributions of the excitation energies are shown in the SI). Note, that compared to previous gas-phase calculations, we have here employed a smaller basis set. The reasons for doing so are twofold: First, to reduce the cost of performing such calculations. Second, the environment effectively reduces the space available for the electronic density of the probe molecules. Thus, using large and diffuse basis sets in embedding calculations without any account of non-electrostatic repulsion could introduce artifacts in the results due to non-physical electron spill-out effects. The spectroscopic properties of the probes inside the membrane have been obtained from MM sampled structures, i.e., structure not optimized at the same level of theory as in gas-phase. Thus, an additional set of gas-phase calculations with these structures was carried out. This allows one to determine the effects of using MM-sampled structures. The results of these calculations are reported in the Supporting Information.

**Table 3:** The lowest excitation energy (in eV and nm) together with the oscillator strength and two-photon absorption cross-section (in GM) of Nile Red and analogs calculated based on either no environment (gas-phase) or the membrane environment (PE).

<table>
<thead>
<tr>
<th>Probe</th>
<th>$\Delta E$ (λ)</th>
<th>$f$</th>
<th>$\sigma^{2PA}$</th>
<th>$\Delta E$ (λ)</th>
<th>$f$</th>
<th>$\sigma^{2PA}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR</td>
<td>3.26 (380)</td>
<td>0.92</td>
<td>64.7</td>
<td>2.87 (431)</td>
<td>0.49</td>
<td>38.31</td>
</tr>
<tr>
<td>1</td>
<td>3.20 (387)</td>
<td>0.93</td>
<td>-</td>
<td>2.81 (441)</td>
<td>0.59</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>3.19 (389)</td>
<td>0.94</td>
<td>-</td>
<td>2.80 (442)</td>
<td>0.62</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>3.25 (381)</td>
<td>0.91</td>
<td>75.2</td>
<td>2.94 (421)</td>
<td>0.51</td>
<td>36.55</td>
</tr>
<tr>
<td>4</td>
<td>3.28 (377)</td>
<td>0.92</td>
<td>-</td>
<td>2.90 (428)</td>
<td>0.55</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>3.09 (401)</td>
<td>0.86</td>
<td>-</td>
<td>2.88 (431)</td>
<td>0.41</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>3.24 (383)</td>
<td>0.94</td>
<td>-</td>
<td>2.83 (438)</td>
<td>0.47</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>3.23 (384)</td>
<td>0.90</td>
<td>-</td>
<td>2.82 (439)</td>
<td>0.50</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>3.19 (388)</td>
<td>0.74</td>
<td>59.2</td>
<td>2.83 (438)</td>
<td>0.43</td>
<td>37.96</td>
</tr>
</tbody>
</table>

The main observations from the data presented in Table 3 are that (i) the excitation energies...
become red-shifted upon introducing the effect of the membrane, (ii) both the oscillator strength and the two-photon absorption become decreased in the membrane. Both of these observations are in complete agreement with the observations made in a previous study of cholesterol probes. Inspection of the results for the excitation energies and oscillator strengths based on the MM optimized geometries (see the Supporting Information) reveal that the red-shift observed in the PE calculations is mainly due to the introduction of the membrane environment. Although a considerable red-shift exists between the DFT optimized and MM sampled structures, the major contribution to this red-shift originates from the presence of the membrane.

Conclusions

In this paper, we have used a combined approach based on electronic structure calculations and MD simulations to gain insight into the optical and mechanical properties of a series of analogs of Nile Red. By introducing push or pull substituents to the parent Nile Red molecule, the optical properties (one- and two-photon absorption) can be improved without significant perturbation of the membrane environment. Of the considered probes, we find that analogs 3 and 8 are very interesting candidates for further exploration. These probes show improved optical properties and have only minor effects on the membrane properties. In addition, they both possess a relatively large change in dipole moment upon excitation, thereby making them good probes concerning measurements of local dielectrics. Based on these calculations, we find that the membrane environment leads to a red-shift in the absorption energies but at the same time to a slight reduction in the one- and two-photon absorption strengths. This decrease is in agreement with the results of a previous study based on analogs of cholesterol.
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Supporting Information Available

Detailed discussion about the optical properties in gas-phase and an overview of the ab-
sorption intensities and energies for all the snapshots considered in the PE calculation are included.
References


Graphical TOC Entry