Structural design of intrinsically fluorescent oxysterols

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Highlights

Intrinsically fluorescent oxysterols are suitable analogs of their natural counterparts.

Extending the conjugated double bond system of such analogs improves their fluorescence properties.

New fluorescent oxysterols with improved one- and two-photon absorption are predicted by theory.
Structural design of intrinsically fluorescent oxysterols

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Abstract

Oxysterols are oxidized derivatives of cholesterol with many important biological functions. Trafficking of oxysterols in and between cells is not well studied, largely due to the lack of appropriate oxysterol analogs. Intrinsically fluorescent oxysterols present a new route towards direct observation of intracellular oxysterol trafficking by fluorescence microscopy. We characterize the fluorescence properties of the existing fluorescent 25-hydroxycholesterol analog 25-hydroxycholestatrienol, and propose a new probe with an extended conjugated system. The location of both probes inside a membrane is analyzed and compared with that of 25-hydroxycholesterol using molecular dynamics simulations. The analogs’ one- and two-photon absorption properties inside the membrane are evaluated using electronic structure calculations with polarizable embedding. Due to predicted keto–enol tautomerisation of the new oxysterol analog, we also evaluate the keto form. Both analogs are found to be good probe candidates for 25-hydroxycholesterol, provided that the new analog remains in the enol-form. Only the new analog with extended conjugated system shows signif-

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significant two-photon absorption, which is strongly enhanced by the presence of the membrane.

Keywords: oxysterol, fluorescence, electronic structure calculation, polarizable embedding, imaging, intracellular transport

1. Introduction

Oxysterols are oxidized derivatives of cholesterol, which despite their low abundance have important biological functions ranging from down-regulation of cholesterol synthesis, activation of cholesterol esterification over stimulation of cholesterol efflux to induction of cell signaling[1]. Oxidation of cholesterol can happen spontaneously or enzyme-catalyzed, and it is often a sign for accumulation of cholesterol beyond the immediate demand of cells. Accordingly, oxysterols act as sensors for excess sterol: side-chain oxidized sterols such as 25-hydroxycholesterol (25-OH-Chol) inhibit the translocation of the sterol-response element binding protein (SREBP)-activating complex (SCAP) from the endoplasmic reticulum (ER) to the Golgi apparatus[2]. This causes a shut-down of expression of enzymes in the cholesterol synthesis pathway and of the receptor for low density lipoprotein (LDL), thereby blocking for de novo synthesis and cellular uptake of cholesterol[3]. At the same time, binding of 25-OH-Chol and other oxysterols to the LXR transcription factors can stimulate expression of proteins within the cholesterol efflux pathway, such as ATP binding cassette transporter 1 (ABCA1) or the related ABCG1, but also of Idol, an E3 ubiquitin ligase that mediates degradation of the LDL receptor[4]. The effect of expressing such proteins is the upregulation of cellular efflux of excess cholesterol and complete shutdown of cholesterol uptake via the LDL pathway. Finally, 25-OH-Chol and other oxysterols are potent activators of acyl-coenzyme A acyltransferase (ACAT), which esterifies excess cholesterol in the ER for storage in lipid droplets[5].

Beside their importance in regulating cholesterol levels in cells by the mechanisms described above, oxysterols are also potent competitors for almost all
known cholesterol binding proteins. This includes the oxysterol-binding protein (OBP) family of proteins in mammalian cells and their homologs in yeast[6, 7], but also well-known endosomal cholesterol transporters such as the Niemann Pick C1 (NPC1) protein or its homolog in the apical membrane of enterocytes and hepatocytes, NPC1-like 1 protein (NPC1L1). Some oxysterols can even act as chaperones to stabilize the three-dimensional structure of otherwise misfolded NPC1 mutant proteins[8]. The biological relevance of this high affinity of oxysterols to known cholesterol transporters is not well known.

Oxysterols also play important roles in cell signalling and activation. A particular example is the sonic hedgehog pathway, in which binding of various oxysterols as well as of cholesterol to smoothened can activate the pathway, as monitored by cilia localization of smoothened and by expression of Gli1-regulated genes[9, 10, 11]. Mutations in this pathway can cause various forms of cancer and are also responsible for developmental abnormalities. Side-chain oxidized sterols such as 25-OH-Chol also regulate synthesis of other lipids, for example sphingomyelin, and trigger re-allocation of the OBP to the Golgi[12, 13, 14]. Some oxysterols, especially ketosterols, are cytotoxic and trigger apoptosis in various cell lines, which has been linked to their effects on lipid packing and phase behaviour in model membranes[15, 16].

Oxysterols bear polar groups such as hydroxyl-, keto- or epoxy groups, which make them more water soluble compared to cholesterol. As a consequence, intracellular trafficking of the oxysterols studied so far has been shown to be much faster compared to cholesterol[17, 18]. Also, due to their higher partitioning into the water phase, side-chain oxidized sterols such as 25-OH-Chol might exist to a significant extent in monomeric form in the aqueous phase of the cytoplasm, which could affect their binding to proteins[19]. In addition, due to their higher water solubility, oxysterols such as 25-OH-Chol, 27-hydroxycholesterol and 24(S)-hydroxycholesterol, but not cholesterol, have been shown to cross the blood-brain barrier, thereby connecting the peripheral with the cerebral sterol metabolism[20, 21].

Despite their importance, knowledge of intracellular location, kinetics and
transport of oxysterols is scarce. This is largely due to the lack of suitable oxysterol analogs, which could for example be monitored by fluorescence microscopy in living cells. Any extrinsic attachment of a fluorescent group would change the water solubility, polarity, size and possibly also the charge of the oxysterol analog compared to its natural counterpart. As the biological effects of small cholesterol modifications are already significant, attaching a dye is not a viable strategy for imaging of oxysterols. One way to overcome this limitation is to use an alkyne-tagged oxysterol, which represents a minimal chemical alteration, leaving the parent oxysterol structure unchanged[22, 23]. In a subsequent click reaction, a fluorescent azide dye is covalently linked to the alkyne, allowing for visualization of the sterol distribution by microscopy. However, this procedure requires cell fixation and a copper-catalyzed click reaction, and, again, the attached dye can change the sterol localization[24].

Another strategy is to make use of intrinsically fluorescent sterols, which bear several conjugated double bonds in the steroid ring system, making the resulting probes slightly fluorescent in the ultraviolet (UV) region. This approach has been extensively followed for analogs of cholesterol and ergosterol, such as cholestatrienol (CTL) and dehydroergosterol (DHE), in spectroscopy and imaging experiments[25, 24]. Detection of these intrinsically fluorescent sterols by microscopy in living cells is possible when using UV-optimized optics and sensitive CCD detectors on a wide-field microscope[24]. Recently, a derivative of 25-OH-Chol, 25-hydroxycholestatrienol (25-OH-CTL), bearing three double bonds in the steroid ring system has been synthesized and characterized in living Chinese hamster ovarian cells[26]. It was found that 25-OH-CTL traffics to the endocytic recycling compartment from the plasma membrane, similar to CTL and DHE, and was able to upregulate LXR-induced protein expression, similar to 25-OH-Chol[26]. Export of 25-OH-CTL from late endosomes and lysosomes required functional NPC1, which is known to bind side-chain oxidized sterols, such as 25-OH-Chol[27, 26]. Thus, design, synthesis and biophysical characterization of intrinsically fluorescent oxysterols lead to a promising approach for future studies of oxysterol trafficking in cells. However, spectroscopic charac-
terization of 25-OH-CTL has not been carried out so far.

In this study, we set up experiments to characterize the excitation and fluorescence properties of 25-OH-CTL in model membranes, and carry on to propose a new probe candidate having four conjugated double bonds, named 25-OH-3a (see Figure 1). The membrane properties and optical properties of this probe as well as the known 25-OH-CTL are examined with computational tools. Using molecular dynamics (MD) simulations, the analogs’ location and orientation inside a membrane are compared with those of 25-OH-Chol in order to assess the suitability of 25-OH-CTL and 25-OH-3a to serve as probes for this important oxysterol. Furthermore, the UV absorption properties of the analogs are evaluated using electronic structure methods. Both one- and two-photon absorption (1PA and 2PA) properties are investigated, and the latter is interesting because excitation via 2PA allows the use of less invasive radiation compared to 1PA (about half the energy) and can thereby be used for live-cell imaging over extended periods of time[25, 28]. Probe 25-OH-3a is inspired by our previous work on cholesterol probes[29, 30]. Here, we also consider the keto form of this probe because we found that probe 3a can undergo keto–enol tautomerization[31], and this is probably also the case for 25-OH-3a. The chemical structures and numbering of the oxysterols treated in this study are given in Figure 1.

2. Methods

2.1. Molecular Dynamics Simulations

The computational approaches for MD simulations and subsequent electronic structure calculations follow closely those described in Refs. 29 and 30, respectively. We perform 220 ns MD simulations of 25-OH-Chol or one of its analogs embedded in a membrane composed of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) lipids, solvated in 0.15 M KCl. Oxysterols are usually present in very small concentrations[1] and we therefore use a mol% of 5.5 oxysterol in this study. Thus, the four systems, i.e. 25-OH-Chol + POPC, 25-OH-CTL + POPC, 25-OH-3a + POPC or 25-OH-3a (keto) + POPC consist of 138 POPC lipids, 8
Figure 1: Chemical structures and numbering of 25-OH-Chol and its analogs.

oxysterols, 5840 TIP3P[32] water molecules, 14 K\(^+\) and 14 Cl\(^-\) ions. The MD simulations were performed with the CUDA[33, 34, 35] version of Amber16[36] using the Lipid14[37] force field for the POPC lipids and the parameters developed by Joung and Cheatham[38] for the ions. Parameters and topologies for the oxysterols were calculated with Antechamber[39] following a Gaussian09[40] calculation, using the RESP[41] formulation for charges and the general Amber force field (GAFF)[42] for the remaining terms.

Energy minimization and equilibration of the membranes were performed as described in ref. 29. Following this, the membranes were simulated for 220 ns in the NPT ensemble using Langevin[43] dynamics propagated with a simple Leapfrog integrator. The Berendsen barostat[44] with a relaxation time of 1.0 ps was used to keep the pressure at 1 atm and the temperature was controlled by the Langevin thermostat[43] with the target temperature set to 303 K and a collision frequency of \(\gamma = 1.0 \text{ ps}^{-1}\). The SHAKE[45] algorithm was used to constrain bonds involving hydrogen and the time step was 2 fs. The first 90 ns of the
220 ns simulation were removed before the analysis, which was performed with AmberTools16[36] and GROMACS[46] version 5.1.2[47]. A frame was extracted every 20 ps from the remaining 130 ns trajectory, rendering 6500 frames for analysis. The area per lipid was calculated with the APL@Voro software,[48] which performs a Voronoi tessellation for a set of selected key atoms—in this case the phosphate atoms of the lipids and the oxygen atoms of the 3α-OH groups of the oxysterols. Block errors[49, 50] given in Table 1 are calculated using the standard formulations implemented in GROMACS.

2.2. UV Absorption Calculations

The electronic structure calculations of the probes inside the membranes were performed as in ref. 30, using the last snapshot from the simulation for each system. The simulation box was centered around each of the eight probes at a time in order to get eight different structures for subsequent absorption calculations for each system. Two snapshots, at 180 ns and at 220 ns, were used for 25-OH-3α + POPC, giving 16 structures for evaluation of absorption properties. The excitation energies and 1PA and 2PA properties were calculated using time-dependent density functional theory (TD-DFT) with the CAM-B3LYP[51] exchange–correlation functional and the 6-311++G** basis set[52] for the probes. The electrostatic and polarization effects of the surroundings were modeled with the effective external field (EEF)[53] extension of the polarizable embedding (PE) model[54, 55, 56]. The vacuum results were calculated on a single geometry optimized at the B3LYP[57, 58, 59]/6-311++G** level of theory using Gaussian09[40], while transition properties were evaluated with CAM-B3LYP using the same basis set.

2.3. Excitation and fluorescence measurements

Appropriate amounts of POPC and 25-OH-CTL were mixed in a glass vial. Solvent was evaporated under N₂ steam, and lipids were dissolved in distilled water to a final concentration of 150 µM. Large unilamellar vesicles (LUVs) were made by extrusion using a 400 nm pore diameter filter and a micro extruder from
Avanti Polar Lipids. Steady-state excitation spectra of 20 mol% 25-OH-CTL were measured using an ISS Chronos spectrofluorometer (Urbana-Champaign, IL). Excitation was measured at an emission wavelength of 370 nm. For the emission spectra, the excitation wavelength was set to 328 nm, and emission was recorded in a wavelength range of 340–500 nm. The slit width was 0.5 mm and no polarizers were used. Both excitation and emission spectra were corrected for the Raman peak of water and normalized to the respective maximal intensity.

For polarization measurements, the excitation polarizer was set to pass only vertically polarized light, whereas the linear emission polarizer was set in either parallel or perpendicular position to the excitation filter. Polarized emission was measured for liposomes containing POPC and either 20 or 40 mol% 25-OH-CTL and in addition for pure POPC liposomes. The latter was necessary to correct for light scattering artifacts on the anisotropy. In fact, we found that without correction, the anisotropy was arbitrarily high, since scattering of the excitation beam is known to increase the anisotropy value [60]. Emission anisotropy corrected for scattering artifacts was calculated using the relation

\[
r = \frac{(I_{VV} - I_{VV}^*) - G(I_{VH} - I_{VH}^*)}{(I_{VV} - I_{VV}^*) + 2G(I_{VH} - I_{VH}^*)}.
\]

Here, \( I_{VV} \) and \( I_{VH} \) are the emission of 25-OH-CTL parallel and perpendicular to the excitation direction, respectively. For pure POPC liposomes, the intensity parallel and perpendicular to the excitation beam is denoted as \( I_{VV}^* \) and \( I_{VH}^* \), respectively. To obtain the G factor, measurements with the excitation polarizer set to pass only horizontally polarized light and the emission filter set either parallel or perpendicular to the first, were conducted using the POPC liposome suspension with the respective concentrations of 25-OH-CTL, giving:

\[
G = \frac{I_{HV}}{I_{HH}}.
\]

3. Results and Discussion

3.1. Membrane Properties

The MD simulations revealed several possible locations for oxysterols inside the POPC bilayer. Figure 2 shows a snapshot from the simulation of 25-OH-
Figure 2: Snapshot from the simulation of 25-OH-Chol in POPC. 25-OH-Chol is shown in a space-filling model and the phosphocholine head groups of POPC in stick representation. Lipid tails, water and ions are omitted for clarity. Color code: C (cyan), H (white), O (red), N (blue), P (yellow). The figure was produced with the Visual Molecular Dynamics program (VMD)[63].

Chol in POPC. This snapshot illustrates the "normal" upright orientation of the oxysterol (1), i.e. located in one of the bilayers and with the 3α-OH group in the lipid headgroup region, and an interfacial orientation (2), where the oxysterol is located in the head group region, perpendicular to the bilayer normal and with both hydroxyl groups available for hydrogen bonding with the lipid head groups. This interfacial orientation has been observed for 25-OH-Chol in previous studies[61, 62]. It is also visible how the 25-OH groups of two oxysterols (1 and 3) in different monolayers are located very closely together, enabling hydrogen bonding with each other in the center of the membrane. Finally, one oxysterol (4) is in a trans-bilayer location and reaches across both leaflets to form hydrogen bonds with the head groups on both sides. This has the effect of locally thinning the membrane.

Table 1 gives average values for the bilayer thickness and area per POPC
Table 1: Average values of membrane properties for the pure POPC bilayer and for the bilayers with 5.5 mol% oxysterol in POPC at 303 K.

<table>
<thead>
<tr>
<th>Oxysterol</th>
<th>Bilayer thickness (nm)</th>
<th>Block error (nm)</th>
<th>Area/lipid (nm²)</th>
<th>Block error (nm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pure*</td>
<td>3.77</td>
<td>0.02</td>
<td>0.66</td>
<td>0.01</td>
</tr>
<tr>
<td>25-OH-Chol</td>
<td>3.85</td>
<td>0.02</td>
<td>0.64</td>
<td>0.01</td>
</tr>
<tr>
<td>25-OH-CTL</td>
<td>3.84</td>
<td>0.01</td>
<td>0.64</td>
<td>0.01</td>
</tr>
<tr>
<td>25-OH-3a</td>
<td>3.78</td>
<td>0.02</td>
<td>0.65</td>
<td>0.01</td>
</tr>
<tr>
<td>25-OH-3a (keto)</td>
<td>3.81</td>
<td>0.02</td>
<td>0.65</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*data taken from ref. 29.

lipid over the 130 ns trajectory together with the respective block errors for a pure POPC bilayer (data taken from ref. 29) and for the POPC bilayers containing 5.5 mol% oxysterol. The residence time of an oxysterol in either of the described locations in the bilayer is on the order of tens of ns, meaning that the simulation time required for complete statistical sampling may be longer than the current simulations. However, we do observe the entire ensemble of orientations of the oxysterols in the membrane, as shown in Figure 2. In general, the presence of oxysterols thickens the membrane compared to the pure POPC membrane, which is also reflected in a decreased area per lipid. The membrane thickness with 25-OH-3a stands out slightly, which can be explained by the fact that it has one or two oxysterols in the trans-bilayer location during most of the simulation, leading to a thinning of the membrane. The average deuterium order parameters for the lipid sn-1 (palmitoyl) and sn-2 (oleoyl) tails are given in Figure 3 for all 5 systems, showing that all oxysterols – including 25-OH-3a – increase the order in the membrane. Figure 4 shows the normalized tilt angle distribution for the steroid ring system (the molecular axis defined to go from C13 through C10) for the different systems. 25-OH-Chol, 25-OH-CTL and 25-OH-3a have very similar distributions where the oxysterols spend the majority of their time in an upright position, but with a shoulder around 80°.
Figure 3: Deuterium order parameters for the $sn$-1 (a) and $sn$-2 (b) segments of POPC in a bilayer with 5.5 mol% embedded oxysterol, and for a pure POPC bilayer at 303 K. The numbering of the POPC segments can be found in the Supporting Information for ref. 29.

representing the portion of oxysterols with both hydroxyl groups in the lipid headgroup region, perpendicular to the membrane normal. The bimodal tilt angle distribution seen for oxysterols was not observed for cholesterol, CTL, DHE or 3a[29, 64, 65], and is caused by the presence of a polar group in the sterol tail. Previous studies have showed similar complex and bimodal tilt angle distributions for other sterols bearing polar groups in the tail[66, 67]. The keto-form of 25-OH-3α also has a small shoulder at 80°, but has its largest peak around 140°. This means that many 25-OH-3α (keto) molecules got inverted, i.e. have rotated around an axis perpendicular to the molecular long axis. As a consequence, they end up having an orientation where the 25-OH group is in contact with the lipid head groups and the carbonyl is located in the center of the membrane. Visual inspection of the trajectory confirms this, and a single incident of a probe switching leaflets to acquire this orientation was also observed (see supplemental movie). The interfacial location is less favored for the keto-form, likely because it has only one hydroxyl group. These findings are in agreement with previous MD simulations of ketosterone, a cholesterol analog.
where the 3α-OH group is replaced by a ketone group, which showed increased tilt angles and flip-flop motions in a membrane[68]. The electron density plotted across the bilayers are shown in Figure 5. The asymmetry in the 25-OH-3a (keto) distribution between the monolayers arises because one probe switched leaflets, as already discussed. We note that the orientation of the oxysterols may be altered in the cellular membrane by the thickening of the bilayer owing to the presence of cholesterol and sphingomyelin. Thus, the proportion of oxysterols which adopt a transbilayer conformation (type 4 in Figure 2) will be smaller for a cellular membrane than for our model POPC bilayer. However, we can conclude that 25-OH-CTL and 25-OH-3a both qualify as suitable probes for 25-OH-Chol based on their locations and orientations in the membrane. This is not the case for the keto form of 25-OH-3a due to its inverted orientation in the membrane.
Figure 5: Electron density plots across the bilayers originating from the whole oxysterol molecule (red), the 3α-OH group of the oxysterol (black), the 25-OH group of the oxysterol (magenta) the PC head group (green), and the carbonyl groups of the PC only (green dashed line). Bin size is 0.25 Å.
Table 2: Average excitation energies ($\Delta E$) in eV and corresponding absorption maxima ($\lambda_{abs}$) in nm together with average oscillator strengths ($f$) and 2PA cross sections ($\sigma^{2PA}$, 1GM = $10^{-49}$cm$^4$photon$^{-1}$s$^{-1}$) for the lowest $\pi \rightarrow \pi^*$ transition of three 25-OH-Chol analogs in vacuum or in the membrane (with 5.5 mol% probes) modeled with PE-EEF. Standard deviations are indicated in parenthesis. All quantities are calculated at the CAM-B3LYP/6-311++G** level of theory.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Model</th>
<th>$\Delta E$ (eV)</th>
<th>$\lambda_{abs}$ (nm)</th>
<th>$f$</th>
<th>$\sigma^{2PA}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-OH-CTL</td>
<td>vac</td>
<td>3.90</td>
<td>318</td>
<td>0.31</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>membrane</td>
<td>3.94 (0.15)</td>
<td>315</td>
<td>0.26 (0.03)</td>
<td>0.13 (0.19)</td>
</tr>
<tr>
<td>25-OH-3a</td>
<td>vac</td>
<td>3.41</td>
<td>364</td>
<td>0.43</td>
<td>1.90</td>
</tr>
<tr>
<td></td>
<td>membrane</td>
<td>3.34 (0.16)</td>
<td>371</td>
<td>0.33 (0.07)</td>
<td>3.90 (2.39)</td>
</tr>
<tr>
<td>25-OH-3a (keto)</td>
<td>vac</td>
<td>3.86</td>
<td>321</td>
<td>0.31</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>membrane</td>
<td>3.69 (0.16)</td>
<td>336</td>
<td>0.24 (0.04)</td>
<td>0.14 (0.27)</td>
</tr>
</tbody>
</table>

3.2. Optical Properties

The 1PA and 2PA properties of the analogs have been calculated for the probes in isolation and inside the membrane (PE-EEF), as described in the Methods section. The results, i.e. excitation energies and corresponding absorption maxima, oscillator strengths and 2PA cross sections are given in Table 2. The membrane values are averages over 8 geometries for 25-OH-CTL and 25-OH-3a (keto), and over 16 geometries for 25-OH-3a. Two snapshots were used in order to have more data points when searching for correlations between the 2PA cross section and the orientation of the 25-OH-3a probes, which will be discussed below. As expected, 25-OH-3a with four conjugated double bonds has a lower excitation energy ($\Delta E$) and larger oscillator strength ($f$) and 2PA cross section ($\sigma^{2PA}$) than the other two probes that have only three conjugated double bonds. Furthermore, insertion of the probes in a membrane reduces the excitation energy as well as the oscillator strength for all probes. This is in agreement with our previous findings for non-oxy sterols[30]. Also in agreement with the previous study is the large increase in 2PA cross sections induced by the membrane for all probes. The 2PA is particularly strong for 25-OH-3a.
No general correlation was found between the orientation or location of the probes in the membrane and their absorption properties (data not shown). Following a single 25-OH-3a molecule every 0.1 ns over the course of 1 ns MD simulation revealed large, more or less uncorrelated fluctuations in the 2PA cross sections, as seen in Figure 6. In each snapshot, the 2PA cross section was calculated both for the probe in isolation (black) and for the probe with the membrane surrounding it (green). It is clear that the large fluctuations in the 2PA cross section is caused by the membrane, indicating that the rapidly changing local orientation of the lipids closest to the probes is decisive for the local electric field, and thereby the 2PA cross section. Similar to our previous study[30], a clear correlation was found between the difference dipole moment of the probes (i.e. the change in dipole moment upon excitation) and the 2PA cross section ($R^2 = 0.86$, data not shown).

In summary, all the examined probes have strong enough 1PA to be used as probes in experimental applications. In particular, 25-OH-3a is predicted to be very useful in experiments relying on a two-photon excitation process.

Figure 6: 2PA cross sections for a 25-OH-3a probe followed in time calculated with (green) or without (black) the membrane present.
Figure 7: A. Excitation (red) and emission (blue) spectra measured for 20 mol% 25-OH-CTL in POPC LUVs. B. Fluorescence anisotropy measured for LUVs having 20 or 40 mol% 25-OH-CTL.

The calculated absorption maximum for 25-OH-CTL embedded in a POPC membrane (315 nm) is in very good agreement with the measured excitation spectrum of LUVs of POPC containing 20 mol% 25-OH-CTL, seen in Figure 7A, red line. The fluorescence maximum is found at ~375 nm (blue line). Fluorescence anisotropy was measured for LUVs of POPC containing different amounts of 25-OH-CTL, 10, 20 or 40 mol% (see Figure 7B). The steady-state fluorescence anisotropy of 25-OH-CTL is close to $r = 0.2$ and fairly constant over a concen-
tration range of 20 to 40 mol% sterol in liposomes (Figure 7B). This is in contrast to what has been observed previously for CTL and DHE[69, 70, 64, 71, 72, 73]. For these sterols, homo-energy transfer (homo-FRET) between the molecules due to decreasing distance and increasing packing at higher compared to lower concentrations in the bilayer has been reported based on a measured decrease in fluorescence anisotropy as function of probe content in the bilayer and theoretical calculations[70, 74]. It is possible that the packing density and/or intermolecular alignment of 25-OH-CTL at 20 and 40 mol% is lower compared to CTL or DHE, which could explain low or even absent homo-FRET (i.e. higher anisotropy) of 25-OH-CTL compared to these fluorescent analogs of cholesterol. This interpretation is fully in line with the MD simulation results (Figure 4).

Since the theoretical value for the angle between the excitation and emission dipoles for 25-OH-CTL is only 0.01 degrees, its limiting anisotropy must be very close to the theoretical limit of $r = 0.4$. Because the measured anisotropy is $r \sim 0.2$ (see Figure 7B), we conclude that some rotational depolarization takes place during the excited-state lifetime of 25-OH-CTL.

From our simulations we calculate the rotational correlation time for an axis defined as the normal to the plane spanned by C6, C10 and C13 of 25-OH-CTL to be 14.7 ns (when fitted to a single exponential function and including data up to 25 ns, where the correlation function evens out), which is significantly longer than the expected excited-state lifetime. For comparison, the corresponding calculated correlation times for CTL and DHE (at 30 mol% and with the same axis and function definitions) are 9.9 ns and 10.1 ns, respectively. These results suggest that intrinsically fluorescent oxysterols have different rotational mobility patterns compared to their parent cholesterol analogues. Note that the exact values for correlation times depend on the definition of the rotation axis and the functional form, as we found different values of the rotational autocorrelation of DHE and CTL in previous studies[64, 29]. Also, the calculated correlation times are not identical to the measurable correlation times in time-resolved fluorescence anisotropy experiments, as the latter are defined based on the transition dipole moment of the sterol molecules and are typically $\leq 1$ ns for CTL and
4. Conclusions

In this study, we have verified the existing 25-OH-Chol analog 25-OH-CTL as a good probe through both spectroscopic characterization and computational analysis. The two methods are in excellent agreement, and we use the computational approach in the design and analysis of new analogs. The new probe candidate 25-OH-3a, provided it stays in its enol form, is shown to be a suitable optical probe for 25-OH-Chol based on its position in the membrane and its absorption properties. It is seen that the conclusions drawn for non-oxy sterols in previous studies[29, 30] are also valid for the 25-OH derivatives. However, 25-OH-3a can undergo keto–enol tautomerization to render the less probe-suitable keto form. Calculations of the relative energies of the keto- and enol-forms of 3a predict an overweight of the keto-form[31], and the same might be expected for 25-OH-3a. However, due to the much reduced 2PA for the keto-form compared to the enol-form only the enol-form will be visualized in two-photon microscopy. Still, due to the different membrane properties of the enol- and keto-forms it is necessary to lock the probe in the enol-form in order to prevent the probe from inverting in the membrane. A locked enol form may be acquired by acetylation of the 3α-OH group, as will be described in future work[31], but the membrane effects and orientation of such a probe should be further investigated.

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