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Polarized Fluorescence and Absorption Spectroscopy of 1,32-Dihydroxy-Dotriacontane-*bis*-Rhodamine 101 Ester. A New and Lipid Bilayer-Spanning Probe

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We report on the properties of 1,32-dihydroxy-dotriacontane-*bis*-rhodamine 101 ester ($Rh101C_{32}Rh101$) in lipid bilayers of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and in liquid solvents. The results are compared with those of rhodamine 101 octadecanyl ester ($Rh101C_{18}$). Both molecules are solubilized in the lipid bilayer and the Rh101 moieties are anchored in the lipid–water interface, so that the electronic transition dipole moments ($S_0 \leftrightarrow S_1$) are oriented preferentially in the plane of the bilayer. At low concentrations of the dyes in lipid bilayers of DOPC, the fluorescence relaxation is single exponential with a lifetime of $\tau = 4.9 \pm 0.2$ ns. The relative fluorescence quantum yield of $\Phi_{C32}/\Phi_{C18} \approx 0.95$ in DOPC vesicles. These results strongly suggest that only a small fraction of the $Rh101C_{32}Rh101$ molecules are quenched, by, for example, intra- or intermolecular dimers in the ground state at mole fractions of less than 0.1% in the lipid bilayers. For $Rh101C_{32}Rh101$ in lipid vesicles, the steady-state and time-resolved fluorescence anisotropies are compatible with efficient intramolecular electronic energy transfer. It is concluded that nearly every $Rh101C_{32}Rh101$ molecule is spanning across the lipid bilayer of DOPC.

KEY WORDS: Bichromophoric fluorescent probe; rhodamine 101; intramolecular electronic energy migration; fluorescence anisotropy; lipid vesicles.

INTRODUCTION

A great number of fluorescent probes have been synthesized for various purposes, as can be realized, for example, by inspecting the *Handbook of Fluorescent Probes*[1] or by reading Refs. 2 and 3. However, the different fluorescent groups used, e.g., rhodamines, coumarines, and pyrenes, are relatively few and it is rather their specific derivatives that add up to a large number of probes. For example, in studies of biomembranes, hydrophobic groups are commonly linked covalently to a fluorophore. Thereby the probes become more or less anchored to the interior or the hydrophobic–hydrophilic interface of lipid bilayers. If the fluorophore is hydrophilic, like, *e.g.*, rhodamine 101, and a long hydrocarbon chain is linked to it, this amphiphilic molecule will incorporate its tail into the bilayer, while the fluorescent moiety remains in the lipid–water interface. The rhodamine 101 octadecanyl ester ($Rh101C_{18}$) is one example of such an amphiphilic probe and these probes are often used in fusion studies of lipid bilayers.[4,5]

In this work, we report on a new bifluorophoric membrane adapted probe, consisting of two rhodamine 101 molecules which are linked to the ends of a long (C_{32}) hydrocarbon chain, as illustrated in Fig. 1. Hereafter we will replace the chemical name of 1,32-dihydroxy-dotriacontane-*bis*-rhodamine 101 ester by the

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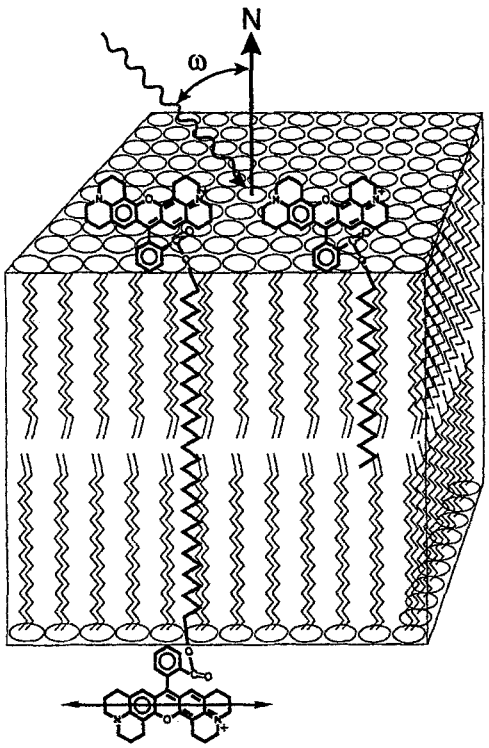

Fig. 1. Schematic illustrating the preferential orientation of the electronic transition dipole (↔) of Rh101C_{18} and Rh101C_{32}Rh101 molecules in lipid bilayers of DOPC. The arrow (↔) indicates the direction of the electronic transition dipole moments ($S_0 \leftrightarrow S_1$) with respect to the chromophore. The size of Rh101C_{18} and Rh101C_{32}Rh101 is approximately scaled to the lipid bilayer thickness. In LD experiments with linearly polarized light the beam impinges at an angle ω of tilt with respect to the normal (*N*) to the oriented lipid bilayers.

acronym Rh101C_{32}Rh101. Provided the distance between the polar head groups (Rh101:s) is sufficiently long, such a molecule can span across a lipid bilayer. Membrane-spanning bifluorophoric probes of this kind are of interest for basic experimental and theoretical studies of electronic energy transfer, regarding both intra- and interbilayer processes. In supramolecular structures[3] they may also be of interest as devices of electronic energy transfer. Another application could be as probes of the lipid packing of membranes.

EXPERIMENTAL

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (U.S.). The purity of the lipid was better than 99% as checked by thin-layer chromatography at our laboratory. Rhodamine 101 octadecyl ester (Rh101C_{18}) was purchased from Molecular Probes (U.S.). The synthesis of 1,32-dihydroxydotriacontane-*bis*-rhodamine 101 ester (Rh101C_{32}Rh101) will be published in a forthcoming paper.

Vesicles were prepared by sonication according to the following procedure. Appropriate amounts of the dry powder of the lipid were dissolved in a mixture of chloroform/methanol (2:1 vol/vol). The solvent was evaporated and the samples were dried at 320 K and 0.1 Torr during at least 2 h. Afterwards, 3 ml of water was added and the suspension was freezed with liquid nitrogen and thawed ten times. The samples were then sonicated eight times in intervals of 5 min. During the sonication the sample was cooled at about 283 K. The sonicator was a Soniprep 150 (MSE Scientific Instruments, England) supplemented with an exponential microprobe. The level of the amplitude used was 10–14 μm.

The lamellar liquid crystalline phase of DOPC was prepared by dissolving the lipid and a probe in a mixture of chloroform/methanol (2:1 vol/vol). The solvent was evaporated and the samples were dried at 320 K and 0.1 Torr during at least 2 h. Weighed amounts of water (20%) were added and the ampules were sealed. The samples were equilibrated for at least 3 d.

The steady-state fluorescence spectra and anisotropies were obtained using a SPEX Fluorolog 112 instrument (SPEX Ind., NJ), equipped with Glan-Thompson polarizers. The spectral bandwidths were 5.6 and 2.7 nm for the excitation and emission monochromators, respectively. The fluorescence spectra were corrected. The fluorimeter was calibrated by using a standard lamp from the Swedish National Testing and Research Institute, Borås.

For the relative fluorescence quantum yield measurements, DOPC vesicles were prepared with Rh101C_{18} and Rh101C_{32}Rh101 under the same conditions and the maximum absorbances were very similar and less than about 0.08. The integrated fluorescence spectra were measured for each probe under identical experimental settings on the fluorimeter and with the excitation and emission polarizers set vertical and at the magic angle, respectively. This procedure was repeated five times and the temperature was kept at 295 ± 0.5 K. The reported values are the mean values.

A PRA 3000 system (Photophysical Research Assoc., Canada) was used for single-photon-counting measurements of the fluorescence decay. The excitation source is a thyratron-gated flash lamp (Model 510C, PRA) filled with deuterium gas and operated at about 30 kHz. The excitation wavelengths were selected by interference filters (Omega/Saven AB, Sweden) centered at 545 nm. The fluorescence emission was observed above 610 nm through a long-pass filter. The maximum ab-

Table I. Fluorescence Lifetime τ and Relative Fluorescence Quantum Yield Φ_{C32}/Φ_{C18} for $Rh101C_{18}$ and $Rh101C_{32}$ Rh101 in Liquids and Suspensions of Small Unilamellar Vesicles of DOPC at 295 K

System	Φ_{C32}/Φ_{C18}	τ (ns)	χ^2
$Rh101C_{18}$ in ethanol	1	4.6 ± 0.1	1.05
$Rh101C_{32}$Rh101 in ethanol	0.90 ± 0.03	4.3 ± 0.1	1.07
$Rh101C_{18}$ in 1-decanol	1	4.3 ± 0.1	0.96
$Rh101C_{32}$Rh101 in 1-decanol	0.86 ± 0.03	4.2 ± 0.1	1.02
$Rh101C_{18}$ in ethanol–water (56.4/43.6 w/w%)	1	4.5 ± 0.1	1.17
$Rh101C_{32}$ Rh101 in ethanol–water (56.4/43.6 w/w%)	0.78 ± 0.03	3.8 ± 0.2[a]	1.45[a]
$Rh101C_{18}$ DOPC vesicles[b]	1	4.9 ± 0.1	1.13
$Rh101C_{32}$Rh101 DOPC vesicles[b]	0.95 ± 0.03	4.8 ± 0.1	1.15

[a] A biexponential fit with $\tau_1 = 4.0$ (91.2%) and $\tau_2 = 1.9 \pm 0.5$ (8.8%) is statistically much better, $\chi^2 = 1.05$.
[b] Mole fraction of probes in lipid bilayer is $< 10^{-3}$.

sorbance of all samples was kept low in order to avoid fluorescence reabsorption.

The time-resolved polarized fluorescence decay curves were measured by repeated collection of photons during 2000 s, for each setting of the polarizers. The emission polarizer was fixed and the excitation polarizer rotated periodically. In each experiment the decay curves $F_{\parallel}(t)$ and $F_{\perp}t)$ were collected, where $\parallel$ and $\perp$ refer to an orientation of the emission polarizer parallel and perpendicular, respectively, with respect to the excitation polarizer. From these a sum curve

$$s(t) = F_{\parallel}(t) + 2G \cdot F_{\perp}(t)$$

and a difference curve

$$d(t) = F_{\parallel}(t) - G \cdot F_{\perp}(t)$$

were calculated. The correction factor G was obtained by normalizing the total number of counts $F_{\parallel}$ and $F_{\perp}$ collected in $F_{\parallel}(t)$ and $F_{\perp}(t)$, respectively, to the steady-state anisotropy r_s as

$$G = (1 - r_s)(1 + 2r_s)^{-1} F_{\parallel}(F_{\perp})^{-1}$$

The fluorescence decay curves were deconvoluted on an IBM-compatible PC, by using a nonlinear least-square analysis based on the Levenberg–Marquardt algorithm.

Linear dichroism (LD) spectra were recorded on a JASCO J-720 supplemented with an Oxley device and the absorption spectra on a GBC 920 spectrophotometer, supplemented with Glan-Thompson polarizers. Details of studying macroscopically aligned lamellar liquid crystals and the interpretation of data are given elsewhere.[6]

RESULTS AND DISCUSSION

The bifluorophoric probe $Rh101C_{32}Rh101$ was studied by means of polarized fluorescence and absorption spectroscopy, when solubilized in lipid vesicles of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), in macroscopically oriented bilayers of DOPC, and dissolved in liquids. The experimental results are compared with those obtained for $Rh101C_{18}$, which is approximately one-half of a $Rh101C_{32}Rh101$ molecule.

The fluorescence and absorption spectra of $Rh101C_{18}$ and $Rh101C_{32}Rh101$ in diluted ethanol solutions are identical within experimental accuracy. The molar absorptivity of $Rh101C_{32}Rh101$ is twice that of $Rh101C_{18}$ [$\varepsilon(\lambda_{max}) \approx 90{,}000\ M^{-1}\ cm^{-1}$ (577 nm) in ethanol at 298 K], as is also to be expected in the absence of strong interactions between the two chromophores. The time-resolved single-photon-counting experiments show that the fluorescence decays of $Rh101C_{18}$ and $Rh101C_{32}Rh101$ are monoexponential with similar lifetimes in ethanol and 1-decanol (see Table I). The lifetime of $Rh101C_{32}Rh101$ in ethanol solutions decreases upon adding water, while it remains the same for $Rh101C_{18}$. The fluorescence quantum yield is slightly lower for $Rh101C_{32}Rh101$ in alcohol and becomes even lower upon adding water. These findings are compatible with an inter/intramolecular aggregation of the Rh101 moieties of the bifluorophoric probe. It is well known[7–9] that xanthene dyes form aggregates at high concentrations in liquid solutions, and that water favors an aggregation. For example, self-quenching due to aggregation of rhodamine 6G in methanol was observed at concentrations higher than ca. 10 mM[7]. Moreover, the absorption spectrum of rhodamine B at submillimolar and millimolar concentrations in water is strongly distorted due to extensive formation of dimers.[8] From the distance, taken to be that of the extended C_{32} chain between the rhodamines of $Rh101C_{32}Rh101$, one can estimate the corresponding *monomeric* concentration to be ca. 10 mM in a liquid. Therefore, one would expect a slightly lower quantum yield of $Rh101C_{32}Rh101$ in alcohols, similar to what has been found for Rh6G.[7] This is indeed what we find (see Table I). However, the fluorescence lifetime of $Rh101C_{32}Rh101$ in ethanol is slightly, but significantly, shorter than that of $Rh101C_{18}$, which is not expected if the quenching is static, and caused by the formation of intra- or intermolecular dimers in the ground state. This result fits better to a dy-

namic quenching process. On the other hand, static quenching processes fit much better with the results obtained for $Rh101C_{32}Rh101$ in 1-decanol. A possible explanation of these different results may be that rate of a reversible dimerization process is comparable to the fluorescence rate in ethanol, while it is much slower in 1-decanol. By adding water to ethanol, the $Rh101C_{32}Rh101$ molecules are forced to aggregate since they are strongly hydrophobic molecules. In such aggregates ground-state dimers of Rh101 may form which could act as excitation traps of free Rh101 moieties in the aggregate. Consequently, the aggregation would imply lower quantum yields and a nonexponential fluorescence decay of $Rh101C_{32}Rh101$, which is indeed what we find.

In DOPC vesicles the lifetimes of $Rh101C_{32}Rh101$ and $Rh101C_{18}$ are essentially the same, while the quantum yield is slightly lower for $Rh101C_{32}Rh101$. Previously, we have found that by increasing the mole fraction of rhodamine B octadecyl ester ($RhBC_{18}$) in vesicles of DOPC, self-quenching of fluorescence occurs at mole fractions greater than ca. 0.5%.[10] This mole fraction corresponds to a local concentration of about 3 m*M*. For $Rh101C_{18}$, very similar results[11] are obtained and the quenching efficiency is 50% at mole fractions of ca. 2% $Rh101C_{18}$ in DOPC vesicles. In present studies, the mole fractions of $Rh101C_{32}Rh101$ and $Rh101C_{18}$ are less than 0.1% and *intermolecular* self-quenching is negligible. For a mole fraction of ca. 2% $Rh101C_{18}$, the estimated average distance between the chromophores is about 3.5 nm, which is about the distance between the Rh101:s of an extended $Rh101C_{32}Rh101$ molecule. Therefore, unless the Rh101 moieties of a $Rh101C_{32}Rh101$ molecule in a DOPC vesicle are somehow hindered, one would expect a substantial self-quenching, which is contrary to what we experimentally find. This finding strongly suggests that the hydrophilic groups Rh101 of the $Rh101C_{32}Rh101$ molecule are preferentially located on *opposite* sides of the lipid bilayer, i.e., they are spanning the bilayer.

The orientation of $Rh101C_{18}$ and $Rh101C_{32}Rh101$ in oriented bilayers of DOPC was studied by linear dichroism (LD) spectroscopy. Lamellar liquid crystals of DOPC were *macroscopically* aligned between quartz prisms so that they form a uniaxial system with its optic axis (*N*) perpendicular to the bilayers. The orientational distribution of chromophoric molecules solubilized in such systems is *uniaxially anisotropic*. For linearly polarized light propagating at an angle ω of tilt to the optic axis, the absorption depends on the direction of the polarization plane (see Fig. 1). The difference in absorption of light polarized in and out of the plane of the bilayer defines LD. LD gives information about the average orientation of the electronic transition dipole moment of the chromophore in terms of an order parameter;

$$S = \int_0^{\pi} \frac{1}{2} f(\beta)\,(3\cos^2\beta - 1)\sin\beta\, d\beta \qquad (1)$$

Here, β is the angle between the electronic transition dipole moment and the normal to the lipid bilayer and $f(\beta)$ is the normalized orientational distribution function. The order parameter can take values of $-1/2 \le S \le 1$, where the limits correspond to a perfect orientation perpendicular and parallel to the normal of the bilayer, respectively. In particular, for an isotropic orientational distribution, $S = 0$. For $Rh101C_{32}Rh101$ and $Rh101C_{18}$ we obtained the order parameters of $S = -0.323 \pm 0.015$ and -0.351 ± 0.017, respectively. The values are similar and mean that the long axis or the transition dipole moment ($S_0 \rightarrow S_1$) of the Rh101 moiety tends to orient in the plane of the lipid bilayers (see Fig. 1). Very similar results were previously found for $RhBC_{18}$ in bilayers of DOPC.[10]

The relative fluorescence quenching quantum yield indicates that a small fraction of the $Rh101C_{32}Rh101$ molecules are statically quenched in DOPC vesicles. This could be understood if this small fraction of $Rh101C_{32}Rh101$ molecules are anchored with both Rh101:s on the *same* side of the lipid bilayer, that is, they are adopt a U-like conformation. For this case, this pair of rhodamines could form an intramolecular dimer, and the relative quantum yield would become lower while the lifetime remains the same. On the basis of the fluorescence quenching data we estimate that ca. 2.5% of the $Rh101C_{32}Rh101$ molecules form ground-state dimers. Their influence on the bandshape of the absorption spectrum or $\varepsilon(\lambda)$ is small, and consequently the ratio between the molar absorption coefficients of the dimer (d) and monomer (m) is $\varepsilon_d(\lambda)/\varepsilon_m(\lambda) < 1$. This agrees qualitatively with previous results on $\varepsilon_m(\lambda)$ and $\varepsilon_d(\lambda)$ for RhB in water.[8] Considering that the order parameter $|S|$ of $Rh101C_{32}Rh101$ is slightly, but significantly lower than that of $Rh101C_{18}$, one may suspect an influence from the dimer absorption on the LD spectrum and on the calculated order parameter. Let us assume that the ratio of $\varepsilon_m(\lambda_{max})/\varepsilon_d(\lambda_{max}) \approx 3$, as obtained for RhB,[8] is relevant also for the Rh101 dimers. Furthermore, assume that the order parameter of the membrane-spanning monomeric $Rh101C_{32}Rh101$ can be taken as that of $Rh101C_{18}$. Then it is possible to estimate an order parameter for the dimer. A straightforward calculation yields values of $S > 1$, which are irrelevent. Therefore, a somewhat different penetration of the Rh101 moieties

in the lipid–water interface is a more feasible explanation of the slightly different S values.

The fluorescence anisotropy is an orientational correlation function, and depends on the rates of molecular reorientations, order parameters, as well as energy transfer/migration processes. At sufficient dilution of $Rh101C_{18}$ in DOPC vesicles (i.e., at mole fraction <0.1%), the influence of donor–donor transfer is negligible, and the $r(t)$ decreases with time solely due to reorientational motions of the Rh101 groups (see Fig. 2). For this case a simple and approximate model of the orientational correlation function is given by

$$r(t) = r_0 [\alpha_1 (1 - S^2) e^{-t/\Phi_1} + \alpha_2 (1 - S^2) e^{-t/\Phi_2} + S^2] \quad (2)$$

where r_0 is the limiting anisotropy, Φ_1 and Φ_2 are rotational correlation times, and $\alpha_1 + \alpha_2 = 1$.

The time-resolved fluorescence anisotropy of $Rh101C_{32}Rh101$ in DOPC at mole fractions <0.1% is shown by the lower graph in Fig. 2. The $r(t)$ is initially much more rapid than that for $Rh101C_{18}$, but they become nearly parallel for times longer than ca. 8 ns. Although not deconvoluted, a visual comparison of the $r(t)$ data suggests that the initially rapid decay observed for $Rh101C_{32}Rh101$ is caused by *intramolecular energy migration*. Qualitatively, it appears that for times $t > 8$ ns the excitation probability is equally distributed between the two Rh101 groups of an $Rh101C_{32}Rh101$ molecule, and that the decay of $r(t)$ is caused by their reorientational motions. For the time region $t \geq 8$ ns and $k_{DD}t >> 1$, where k_{DD} denotes the rate of intramolecular donor–donor transfer, the fluorescence anisotropy can be written as

$$r(t) = r_0 [\alpha_1 (\frac{1 + \rho_0}{2} - S^2) e^{-t/\Phi_1} + \alpha_2 (\frac{1 + \rho_0}{2} - S^2) e^{-t/\Phi_2} + S^2] \quad (3)$$

In Eq. (3), ρ_0 denotes the initial contribution to the orientational correlation function of secondary excited molecules and it takes values of $0 \leq \rho_0 \leq 1$. Equations (2) and (3) predict the same shape of the dynamic part of $r(t)$. This is indeed what we find by fitting Eqs. (2) and (3) with *one* exponential function to the time region of 8–35 ns. We obtain a rotational correlation time of $\Phi = 11 \pm 2$ ns for both compounds. Hence, the reorientational motions of the rhodamines in $Rh101C_{18}$ and $Rh101C_{32}Rh101$ are very similar. The amplitude of $r(t)$ is essentially reduced by a factor of two, as is also expected from a comparison of Eqs. (2) and (3), when S^2 is small. The plateau region of $r(t) = r_0 S^2$ is reached

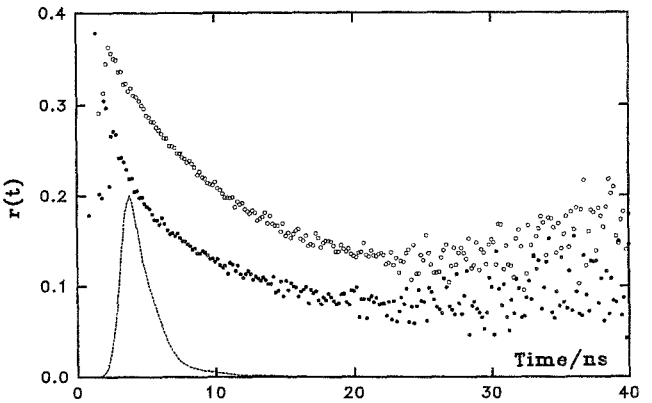

Fig. 2. Fluorescence anisotropy $r(t)$ of $Rh101C_{18}$ (○) and $Rh101C_{32}Rh101$ (●) in vesicles of DOPC at 296 K. The mole fraction of probe is 5×10^{-4}. Note $r(t)$ shown is not corrected (deconvoluted) for the instrumental response function (- - -).

for neither of the probes on the time scale of the experiment. More details concerning Eq. (3) and a complete model including intramolecular energy migration as well as an analysis of the experimental $r(t)$ will be the subject of a forthcoming paper.

The bolaform amphiphile dipotassium hexadecanedioate in water has been studied by means of NMR spectroscopy.[12,13] It was concluded that these molecules form a liquid crystalline phase which is lamellar. In a lamellar phase monoamphiphilic molecules form infinite bilayers, and these are stacked into a multibilayer structure. The liquid crystal of dipotassium hexadecanedioate and water can be visualized as resulting from monoamphiphilic molecules (potassium octanoate) which have been dimerized by covalently linking the ω-methyl groups of the alkyl chains. Recently, the structure of tetraether bolaform amphiphiles (with different hydrocarbon chains) in water was studied by Thompson *et al.*[14] Their data suggest that the bolaform amphiphiles span a monolayer-type membrane, and that the U-shaped bolaform configuration is energetically disfavored. Furthermore, the caroviologens, which are symmetric *all-trans* polyenes with charged end groups, orient preferentially perpendicular to a lipid bilayer (i.e., parallel to N in Fig. 1), provided that their length matches or exceeds the thickness of the lipid bilayer.[15] In the present work the alkyl chain length of $Rh101C_{32}Rh101$ is delibrately chosen to match the thickness of a DOPC bilayer, i.e., 38 Å.[16] Taken together, these studies of bolaform amphiphiles and caroviologens and our studies of $Rh101C_{32}Rh101$ and $Rh101C_{18}$ in DOPC vesicles strongly suggest that most of the $Rh101C_{32}Rh101$ molecules are spanning across the lipid bilayer, while the

polar Rh101 groups are anchored in the lipid–water interface.

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