On the origin of the dynamic Stokes shift of Laurdan molecules in lipid membranes*

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Frequency-resolved time correlated single photon counting experiments have been performed to investigate the time scales and molecular mechanisms for solvation dynamics of 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) embedded in liquid-crystalline bilayers of unilamellar dimyristoyl-phosphatidylcholine (DMPC) vesicles. The reconstructed fluorescence spectra as a function of time exhibit a pronounced double-exponential Stokes shift, which is strongly correlated with the rotational diffusion dynamics of the chromophore as evidenced by experiments on transient fluorescence depolarization.

Key words: solvation dynamics, lipids, Stokes shift, solvent reorganization.

Time-dependent solvation phenomena[1] are of tremendous importance for charge and mass transport across biological boundaries, whether it occurs as passive permeation or is mediated by membrane-bound proteins or ion channels. This is because the dynamics of transmembrane transport are ultimately governed by the temporal-structural response through which the membrane accommodates to the dynamically evolving charge distribution associated with the transported molecule. However, despite the major role of these factors in transport processes and information exchange between biological cells or intra-cellular compartments, our current understanding of the time scales and molecular mechanisms of the dynamic membrane solvation is only rudimentary. This is because until today, such dynamics have neither been explored by unambiguous bio-physical-chemical experiments nor been retrieved from existing molecular dynamics computer simulation.

The dynamic solvation in bulk liquids is usually measured using the time-dependent Stokes shift of fluorophores following switching of their charge distribution due to ultrafast optical excitation.[2—4] Unfortunately, these experiments on dynamic membrane solvation are hampered by the inherent roughness of the membrane surface and the ill-defined spatial transition from the inner core of the biological self-assembly to the bulk aqueous phase located at an unknown distance above the hydrophilic membrane residues.[5,6] Hence, an accurate knowledge of the chromophore location within the membrane is required to draw quantitative conclusions from such time-resolved solvation experiments.

Recently, we have applied a femtosecond nonlinear optical coherence experiment (so-called 3PEPS technique) to enable the first real-time monitoring of interface-specific solvation on time scales between 10 fs and 1 ns.[7,8] A polar cyanine dye was used as an optical chromophore, which was noncovalently anchored to the aqueous interface of unilamellar phospholipid vesicles by means of long-chain alkyl residues buried deeply into the membrane core. Our efforts clearly demonstrated the expected disruption of the water hydrogen-bond network by the polar lipid head groups.[8] This membrane-induced perturbation of the short-range structural order of water results in a pronounced deceleration of the hydration dynamics already on femtosecond time scales, as compared with the bulk aqueous phase. Surprisingly,[7] the ultrafast solvation response due to the bulk-like H-bond network of water was recovered at sufficiently low temperatures by cycling through the various thermodynamic phases of the lipid membrane. This finding may be related to the prevalence of high-temperature polymorphic forms of lipid bilayers in living organisms

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Here we studied the dynamic Stokes shift of the optical chromophore, 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) anchored to a bilayer of unilamellar dimyristoyl-phosphatidylcholine (DMPC) vesicles. This optical chromophore and related naphthalene derivatives, first designed by Weber,[9] are often used to study solvent relaxation in lipidic and micellar environments.[10–17] The photochemical and photophysical properties of Laurdan as a membrane probe have recently been reviewed.[18] In particular, the dynamic Stokes shift and dipolar solvent relaxation in lipid membrane environments have been attributed to a relatively small number of water molecules in close proximity of the naphthalene moiety of the chromophore molecule.[13] According to a different interpretation of the time-dependent emission spectra,[17,19] the initially prepared "locally excited" (LE) state evolves into a charge transfer (CT) state upon conformational twisting, resulting in an electronic decoupling of the dimethylamino group from the presumably planar 6-naphthoxy moiety, similar to the formation of the well-known twisted intramolecular charge transfer (TICT) state. Later on, the CT state is stabilized predominantly upon solvent reorganization. In this paper, we readdress the origin of the time-dependent Stokes shift by comparing it to the dynamics of fluorescence depolarization.

Experimental

Multilamellar vesicles of dimyristoyl-phosphatidylcholine (DMPC) in an aqueous solution were prepared and labeled by Laurdan according to standard procedures.[12] An extrusion through polycarbonate filters (100-nm pore diameter, Avestin) gave labeled unilamellar vesicles according to a protocol described previously.[20] Laurdan was purchased from Molecular Probes and the lipid, DMPC, was obtained from Fluka. All solvents employed for sample preparation were spectroscopic grade and were used without further purification. Steady-state absorption spectra of the samples were measured on a Shimadzu UV-160 UV/VIS spectrophotometer. Excitation and emission spectra were recorded on a Spex, Fluorolog 3 fluorescence spectrometer.

Time resolved fluorescence and fluorescence depolarization measurements were performed with a time-correlated single-photon-counting (TCSPC) setup using a TCC 900 plug-in data acquisition card (Edinburgh Instruments). Excitation pulses at 400 nm were provided by doubling the frequency of a home-built mode-locked Ti-sapphire laser whose repetition rate was reduced to 1.9 MHz by an external fused silica acousto-optic pulse picker. The detection system consisted of a photon counting photomultiplier (Becker & Hickl, PMH-100-1) attached to the exit slit of a 0.2-m Czerny-Turner monochromator. Further, the fluorescence was collected from the thermostatic sample cell (Hellma, optical path 1 mm) in the backward direction, passed through a 1-mm thick color glass filter (Schott, GG 420), and imaged through a Glan-Taylor polarizer onto the entrance slit of the monochromator. The fluorescence polarization plane was set to be parallel to the grooves of the monochromator grating to avoid artifacts originating from Wood's anomalies. The polarization of the excitation pulses could be adjusted relative to the detector polarization by rotating a zero-order half-wave retardation plate.

The temporal instrument response function (~230 ps full width at half maximum) was recorded at multiple detection wavelengths by measuring the fluorescence decay for a $0.33 \cdot 10^{-3}$ mol L^{-1} solution of 4-[4-(dimethylamino)styryl]-1-methyl-pyridinium iodide in methanol whose emission was quenched by adding an equivalent amount of potassium iodide. The slit widths of the monochromator were set for a frequency resolution of about 4 nm. The spectral sensitivity function, S(ν), of our setup was determined by recording, in the same geometry, the standard emission from a halogen white light source (Osram 6318, 10 W, 6 V).

Fluorescence photons were counted for identical accumulation times with the polarization plane of the excitation light oriented parallel, perpendicular, and at the magic angle (*i.e.,* 54.7°) relative to that of the sample emission. Great care was taken to ensure that the maximum count rate in the parallel direction never exceeded 19 kHz. The first two measurements provided the following expression for the transient fluorescence anisotropy, $r(t)$:

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)}, \qquad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are the accumulated photon counting histograms polarized parallel and perpendicular with respect to the excitation pulses.

The magic angle histograms $I(\tilde{\nu},t)$ collected at the detection frequency (in wave number units) $\tilde{\nu}$ were used to reconstruct the time-resolved emission spectrum $\sigma(\tilde{\nu},t)$ based on the relation $\sigma(\tilde{\nu},t) = I(\tilde{\nu},t)/S(\tilde{\nu})$. The stationary emission spectra $\sigma(\tilde{\nu})$ calculated from the relation

$$\sigma(\tilde{\nu}) = \frac{1}{S(\tilde{\nu})} \int_0^{\infty} \mathrm{d}t I(\tilde{\nu},t) \qquad (2)$$

were in excellent agreement with experimental steady-state spectra.

Results and Discussion

Dynamic Stokes shift

A complete set of magic angle fluorescence histograms depending on time and the detection wavelength is shown in Fig. 1 as a gray shaded contour diagram. Kinetic traces were recorded for detection wavelengths of $400 \le \lambda \le 615$ nm in 5-nm increments. The Laurdan concentration was 10 mmol L^{-1} at a dye : lipid molar ratio of 10^{-3}. The temperature was 60 °C; at this temperature, the liquid-crystalline phase of the lipid bilayer is thermodynamically stable.

The dynamic Stokes shift, *i.e.*, the time-dependent shift of the emission spectrum to larger detection wavelengths can be clearly seen in Fig. 1. This shift

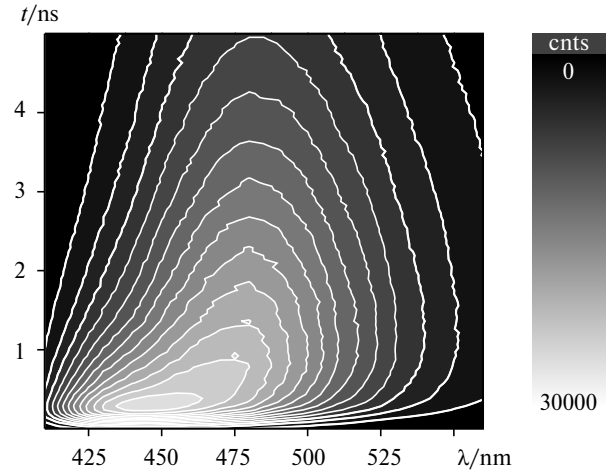

Fig. 1. Gray scale contour diagram of the time dependence of the fluorescence spectrum of Laurdan embedded in unilamellar vesicles of DMPC in an aqueous solution at 60 °C.

can be quantified by calculating, from the time-resolved emission spectrum, the time-dependent first spectral moment

$$\alpha^{(1)}(t) = \langle \tilde{\nu}(t) \rangle = \int_{-\infty}^{\infty} \tilde{\nu} \mathrm{d}\tilde{\nu} \sigma(\tilde{\nu}, t) \Big/ \int_{-\infty}^{\infty} \mathrm{d}\tilde{\nu} \sigma(\tilde{\nu}, t), \qquad (3)$$

corresponding to the mean emission wavelength, $\langle \tilde{\nu},(t) \rangle$, or the "spectral center of mass" as a function of time after photoexcitation. To facilitate such a moment analysis, the sensitivity corrected raw data, $\sigma(\tilde{\nu},t)$, were fit phenomenologically to a superposition of two dynamically evolving Gaussian spectral line shapes

$$\sigma(\tilde{\nu}, t) = \sum_i A_i(t) \exp\left[-\frac{2}{\ln 2} \left(\frac{\tilde{\nu} - \tilde{\nu}_i^0(t)}{\delta\tilde{\nu}_i(t)} \right)^2 \right], \qquad (4)$$

whose time-dependent mean frequency is given by the simple expression

$$\langle \tilde{\nu}(t) \rangle = \sum_i A_i(t) \delta\tilde{\nu}_i(t) \tilde{\nu}_i^0(t) \Big/ \sum_i A_i(t) \delta\tilde{\nu}_i(t), \qquad (5)$$

where $A_i(t)$, $\tilde{\nu}_i^0(t)$, and $\delta\tilde{\nu}_i(t)$ are the time-dependent amplitudes, center-frequencies, and full widths at half maximum for individual components *i*.

The time-dependent first moment of Laurdan in DMPC at a temperature of 60 °C derived in this way is shown in Fig. 2.*

It is clear from Fig. 2 that the Stokes shift decays on at least two distinct time scales. The time dependence of the first moment was described by fitting the data to a double exponential decay (Fig. 2, solid curve)

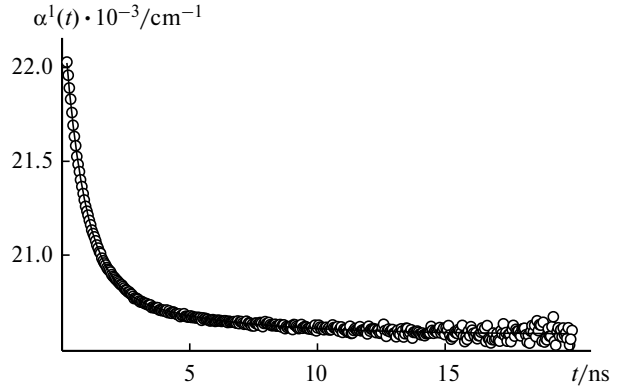

Fig. 2. Time dependence of the spectral center of mass of Laurdan in DMPC at 60 °C: the points correspond to the experimental data and the solid curve is the least-squares fit of the data to a doubly exponential decay according to Eq. 6.

$$\tilde{\nu}(t) = \tilde{\nu}(\infty) + [\tilde{\nu}(0) - \tilde{\nu}(\infty)] \sum_j f_j \exp(-t/\tau_j), \qquad (6)$$

where the experimentally resolved Stokes shift magnitude, $[\tilde{\nu}(0) - \tilde{\nu}(\infty)]$, is equal to the sum over the amplitudes, $\tilde{\nu}_j(0)$, of the individual components, *j*, with the fractional amplitudes $f_j = \tilde{\nu}_j(0)/\Sigma_j \tilde{\nu}_j(0)$ and the correlation times τ_j. The static spectral offset, $\tilde{\nu}_j(\infty)$, can be identified in Fig. 1 as the asymptotic long-time limit of the dynamic Stokes shift.

It should be stressed that this static spectral offset is not necessarily equal to the first spectral moment of the steady-state emission spectrum. This is because the environment may be unable to fully relax within the finite lifetime of the optical chromophore (see also Eq. (2) describing the relationship between the linear and dynamic emission). A nonlinear least-squares analysis of the experimental data (see Fig. 2) yields a value of 20578 cm^{-1} for $\tilde{\nu}_j(\infty)$ and a value of 22360 cm^{-1} for the initial Stokes shift, $\tilde{\nu}(0)$, of Laurdan in DMPC at 60 °C.

Since no correction for the finite time resolution has been made so far, the experimentally resolved Stokes shift magnitude, $[\tilde{\nu}(0) - \tilde{\nu}(\infty)]$, of 1782 cm^{-1} does not correspond to the total dynamic Stokes shift that would result from "impulsive" optical excitation of the chromophore. Therefore, we refrain at this stage from extracting a normalized correlation function, as is commonly done to characterize the solvation dynamics in condensed environments. In addition, we furthermore emphasize that identifying the spectral position, $\tilde{\nu}(0)$, of the emission spectrum at time $t = 0$ is not at all a trivial task, because it is very difficult to unambiguously distinguish between the solvation dynamics and intramolecular vibrational relaxation dynamics even if the pure electronic 0—0 transition for the chromophore is known.[3] The correlation times

* The full analysis of the moments including detailed temperature dependence of the solvation dynamics will be the subject of a forthcoming article.

Table 1. Parameters of the doubly exponential least squares analysis of the experimental data on the dynamic Stokes shift and the fluorescence anisotropy for Laurdan in DMPC at 60 °C

Process	f_1	τ_1/ns	f_2	τ_2/ns
Dynamic Stokes shift	0.830	0.77	0.170	4.36
Fluorescence depolarization	0.725	0.72	0.275	4.40

and the fractional amplitudes of the least squares fit are compiled in Table 1.

Fluorescence depolarization

Figure 3 shows the fluorescence anisotropy for Laurdan in DMPC at 60 °C obtained from two measurements with the excitation polarization either parallel or perpendicular to the collected light. The emission was detected at 490 nm, which corresponds to the wavelength of the maximum fluorescence intensity of the steady-state spectrum. Again, the decay of the transient anisotropy seems to occur on two distinct time scales, as in the case of the dynamic Stokes shift. As was done previously, we fit the data to a double exponential decay according to the relation

$$r(t) = r(\infty) + [r(0) - r(\infty)]\sum_j f_j \exp(-t/\tau_j). \qquad (7)$$

In analogy to the Stokes shift, the anisotropy, $r(t)$, is decomposed into a static term (the long-time limiting value), $r(\infty)$, and a time-dependent term, whose magnitude, $[r(0) - r(\infty)]$, is equal to the sum over the amplitudes, $r_j(0)$, of the individual components, j, with the fractional amplitudes $f_j = r_j(0)/\Sigma_j r_j(0)$ and the relaxation times τ_j. A nonlinear least-squares fit yields 0.008 for $r(\infty)$ and 0.353 for $r(0)$. The obtained fractional amplitudes and time constants are listed in Table 1.

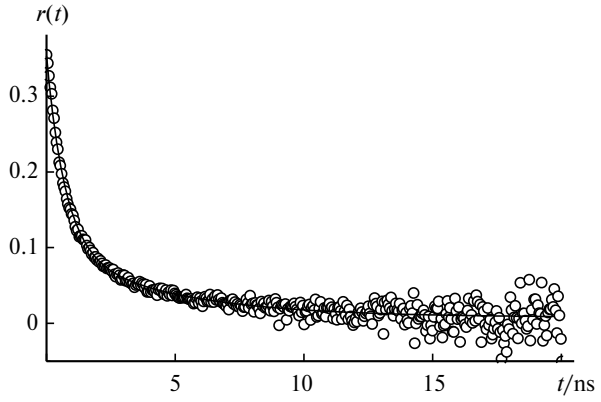

Fig. 3. Decay of fluorescence anisotropy of Laurdan in DMPC at 60 °C: the points correspond to the experimental data and the solid curve is the least-squares fit of the data to a doubly exponential decay according to Eq. 7.

The rationale behind the functional form (Eq. (7)) for the fluorescence anisotropy is known as the "wobble in a cone" model for unrestricted rotational motion about a principal molecular axis which in turn fluctuates randomly (wobbles) about an overall supra-molecular director (*e.g.,* the membrane normal).[21] From Eq. (7), an order parameter, $S = \sqrt{r(\infty)/r(0)}$, can be derived which is related to the cone angle θ (*i.e.*, the angle spanned by the intramolecular axis and the director) by the relation $S = \cos\theta(1 + \cos\theta)/2$. The experiment gives $S = 0.151$ and hence, $\theta = 76°$, *i.e.*, in the liquid crystalline phase, the chromophore rotates diffusively almost as if the director were absent.

Upon closer examination, the data of Table 1 clearly demonstrate that the decay of the fluorescence anisotropy is well correlated with the dynamic Stokes shift. The time constants are identical within the accuracy of the experiment; moreover, the relative amplitudes of the fast and slow contributions to the double exponential fit are very similar. This agreement between two seemingly unrelated experiments requires special attention.

Origin of the Stokes shift

It was previously suggested[17,19] that the excited state dynamics of Laurdan involves an intramolecular charge transfer, which is facilitated by a relative twisting motion between the dimethylamino and naphthalene moieties. Using the maximum entropy analysis of spectrally resolved fluorescence kinetics of Laurdan in polar solvents, the time constant for the charge transfer reaction has been determined[19] to be about several tens of picoseconds, while the remaining slower dynamics were assigned to the subsequent Stokes shift due to solvent reorganization. Similarly, in lipid membranes, the same charge transfer state was believed to be formed within several hundred picoseconds.[17] In these cases, even in the absence of any solvent reorganization, one can expect that the spectrally integrated fluorescence would decay multi-exponentially, because it is quite likely that the individual oscillator strengths of the LE and CT states are significantly different. A small time constant of the expected multi-exponential decay corresponds to the inverse charge transfer rate coefficient, while the slow contributions arise from the finite fluorescence lifetimes of the LE and CT states and from solvent reorganization dynamics if such dynamics are present.

In order to find out whether the depolarization of fluorescence reflects only the reorientational motion of the chromophore or also the scrambling of the orientation of the transition dipole due to additional excited-state relaxation pathways such as charge transfer, we cal-

culated the frequency-integrated fluorescence decay using the data shown in Fig. 1. Indeed, we found that the total fluorescence of Laurdan in DMPC at 60 °C decays strictly mono-exponentially with a time constant of 3.24 ns. This result leads to the conclusion that under these conditions, the intramolecular charge transfer reaction of the excited state is either considerably faster than our time resolution or it deactivates the optically prepared emitting state on a time scale slower than 3.24 ns. In addition, we did not find an isosbestic point, which could confirm unambiguously the existence of two emitting species (*i.e.*, LE and CT) on picosecond to nanosecond time scales. Hence, we believe that on the time scale $0.2 \le t/\text{ns} \le 3\,3$, no charge transfer reaction takes place in the excited state of Laurdan in lipid membranes and that the entire spectro-temporal response is brought about by the solvent reorganization. Any preceding charge transfer reactions are entirely irrelevant to the discussion of the Stokes shift, as they are virtually completed within the excitation pulse. In other words, within our time resolution, the pump pulse effectively generates the CT state in a quasi-instantaneous fashion. Therefore, the fluorescence anisotropy is solely due to the rotational motion of the entire chromophore and not to a reaction-induced rotation of its transition dipole in the molecular frame.

Thus, the striking similarity between the fluorescence depolarization and the dynamic Stokes shift strongly suggests that dipolar solvation of Laurdan in DMPC bilayers at 60 °C is dictated by solute reorientation rather than by solvent reorganization. However, this interpretation of the time-dependent Stokes shift has been previously dismissed in the literature for the following reasons.[18]

(*i*) It was argued[13] that reorientation of the probe fluorescent moiety should be sensitive to the charge of the polar head group. If the solute reorientation were indeed the primary cause for solvation processes in lipids, the dynamic Stokes shift would also be sensitive to the nature of the phospholipid head group. Yet, fluorescence excitation and dispersion spectra of Laurdan measured in a variety of different lipids and lipid mixtures as well as under different pH were reported to be essentially identical.[13] Nevertheless, generalized polarization data (see Ref. 13) as a function of lipid composition do show some considerable dispersion depending on the head group of the lipids, especially for the high-temperature liquid-crystalline phase. It should be emphasized that despite being quite instructive, linear spectra such as excitation and emission spectra do not contain any conclusive information regarding the underlying line broadening dynamics. Equation (2) may imply that entirely different Stokes shift dynamics can result in identical linear emission spectra. In fact, this notion is at the center of a number of experimental and theoretical studies of line broadening phenomena and solvation dynamics in molecular liquids using third-order nonlinear-optical spectroscopies such as photon echoes and related four-wave mixing processes.[22—24] In addition, we are not aware of any time-resolved fluorescence anisotropy data in the literature which could have unambiguously ruled out a possible connection between the time-dependent Stokes shift and the rotational diffusion dynamics of the solute.

(*ii*) It was argued[18] that if rotational diffusion is responsible for the Stokes shift, different orientations of the molecular dipole of the probe within the lipid bilayer would be displayed in the polarization emission spectrum (PES). In other words, a correlation is expected between the position of the emission spectrum of the chromophore and the angle of the chromophore relative to the polarization plane of the excitation light. Indeed, the emission polarization markedly decreases with an increase in the emission wavelength but only up to ~460 nm, while above this wavelength, the polarization levels off.[12,18] In particular, the weak wavelength dependence of the polarization above 460 nm was taken as a clear evidence for negligible contributions of the rotational motion to the time-dependent Stokes shift.[18] However, it is not entirely clear how one can possibly map the PES onto an angular position of the chromophore (*e.g.*, with respect to the membrane normal). In fact, the exact value of the fluorescence polarization at a given wavelength λ is proportional to the temporal integral over the decay of the fluorescence anisotropy, weighed by the temporal fluorescence intensity profile at the magic angle. Hence, the decrease in the emission polarization with increasing emission wavelength can be understood qualitatively in quite simple terms of rotational motion.

In the blue spectral region, the dynamic Stokes shift will result in a rapid decay of the magic angle fluorescence and will therefore enhance the early-time portion of the time-dependent anisotropy. In contrast, in the red spectral region, the dynamic Stokes shift will initially increase the magic angle fluorescence intensity, thus enhancing the late-time portions of the decay of the anisotropy. Consequently, a larger emission polarization is detected in the blue spectral region as compared to the red region. The precise functional dependence of the polarization decay on the emission wavelength will depend on finer details of the Stokes shift dynamics such as the evolution of the first and higher moments of the fluorescence spectrum with time.

Certainly, the data presented here do not suffice to prove ultimately that the chromophore reorientation is responsible for solvation dynamics in lipid membranes. However, the close similarity of the two experiments, *i.e.*, the dynamic Stokes shift, on the one hand, and fluorescence depolarization, on the other hand, is at least highly suggestive. Further experiments including the detailed temperature dependence and a full moment analysis will be presented in the near future.

In conclusion, we have studied the dynamics of solvation of the well-known naphthalene chromophore, Laurdan, embedded in unilamellar vesicles of DMPC at a temperature corresponding to the liquid-crystalline phase of the lipid bilayer. A comparison of the resulting time dependence of the first moment of the chromophore emission spectrum with complementary data on the fluorescence anisotropy decay of the chromophore under the same experimental conditions strongly implies that the solvation dynamics of this chromophore is governed by solute rotational reorientation inside the membrane rather than structural reorganization of the membrane constituents themselves. Further studies on the phase behavior of the Stokes shift dynamics and the fluorescence anisotropy are needed to conclusively connect these two seemingly unrelated experiments.

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