Langmuir
The ACS Journal of Surfaces and Colloids

FEBRUARY 5, 2002
VOLUME 18, NUMBER 3

Letters

On What Time Scale Does Solvent Relaxation in Phospholipid Bilayers Happen?

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Received August 21, 2001. In Final Form: November 19, 2001

Time-resolved emission spectra of seven fluorescent probes in egg-phosphatidylcholine bilayers have been investigated. About 90% of the solvent relaxation monitored by the headgroup labels Prodan, Laurdan, and Patman and by the backbone label 2-AS can be captured with an instrument providing subnanosecond time resolution. In comparison to 2-AS, the transient red-shift of 9-AS is characterized by a larger contribution of a picosecond process and by slower nanosecond dynamics. The major contribution to solvent relaxation probed by C_{17}DiFU and Dauda is faster than the ultimate time resolution of the experiment; those chromophores appear to be located within the external interface of the bilayer.

Introduction

During the past years, solvent relaxation monitored by time-resolved fluorescence measurements has become an extremely useful method in membrane research.[1] It has been shown that suitable fluorescent dyes allow for direct observation of viscosity and polarity changes in the vicinity of the probe molecule which can be intentionally located in the hydrophobic backbone or in the hydrophilic headgroup region of the phospholipid bilayer. The benefit of this approach in biomembrane research has been demonstrated in several publications.[1−5] The development of ultrafast spectroscopic methods has led to an accurate description of the solvent relaxation process for a large variety of isotropic polar solvents. At ambient temperatures, a typical solvent relaxation process in solution starts with a fast inertial (librational) motion on the 50−500 fs time range, followed by rotational and translational diffusion occurring on the pico- to subnanosecond time scale.[6,7] On the other hand, it has been demonstrated[1−5,8,9] that a substantial part of solvent relaxation monitored by dyes associated with phospholipid bilayers occurs on the nanosecond (ns) time scale. A quantitative description of the solvent relaxation in bilayers, however, is still missing, which represents a limitation for more frequent applications of this approach in membrane studies. Thus, we

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(1) Hof, M. In *Solvent Relaxation in Biomembranes (Applied Fluorescence in Chemistry, Biology, and Medicine)*; Rettig, W., Ed.; Springer-Verlag: Berlin, 1999; p 439.
(2) Hutterer, R.; Schneider, F. W.; Sprinz, H.; Hof, M. *Biophys. Chem.* **1996**, *61*, 151−160.
(3) Hutterer, R.; Schneider, F. W.; Hermens, W. T.; Wagenvoord, R.; Hof, M. *Biochim. Biophys. Acta* **1998**, *1414*, 155−164.
(4) Hutterer, R.; Schneider, F. W.; Lanig, H.; Hof, M. *Biochim. Biophys. Acta* **1997**, *1323*, 195−207.
(5) Hutterer, R.; Schneider, F. W.; Hof, M. *J. Fluoresc.* **1997**, *7*, 27−33.
(6) Horng, M. L.; Gardecki, J. A.; Papazyan, A.; Maroncelli, M. *J. Phys. Chem.* **1995**, *99*, 17311−17337.
(7) Jimenez, R.; Fleming, G. R.; Kumar, P. V.; Maroncelli, M. *Nature* **1994**, *369*, 471−473.
(8) Krishna, M. M. G. *J. Phys. Chem. A* **1999**, *103*, 3589−3595.
(9) Kumar Pal, S.; Sukul, D.; Mandla, D.; Bhattacharyya, K. *J. Phys. Chem. B* **2000**, *104*, 4529−4531.

10.1021/la011337x CCC: $22.00
Published on Web 01/10/2002

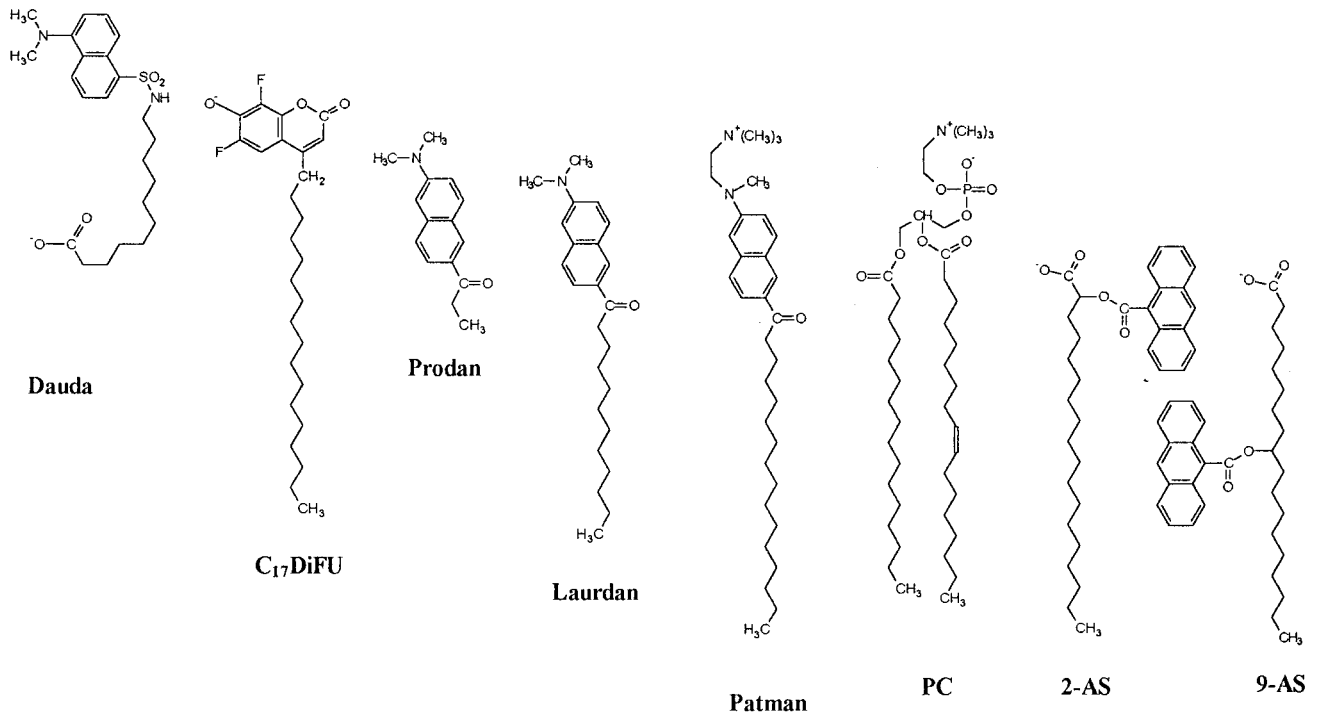

Figure 1. Molecular structures of the investigated dyes and their schematic positions relative to PC. Please note that C_{17}DiFU at pH = 7.4 and incorporated in SUVs is deprotonated. One criterion for choosing these dyes has been a rather defined localization of those dyes within the bilayer. An exception in this respect might be Prodan.

decided to monitor solvent relaxation response of seven membrane labels at different locations within or near the egg-phosphatidylcholine (PC) bilayer (see Figure 1) in the liquid-crystalline phase[10] in order to answer the question to what extent the solvent relaxation monitored by these labels can be detected with single photon counting (SPC) equipment providing subnanosecond time resolution. To estimate the extent of the solvent relaxation process the experiment has captured, we use the time-zero ($t = 0$) spectrum estimation introduced by Fee and Maroncelli[6,11] as well as the time-evolution profile of the spectral halfwidths (full width at half-maximum, fwhm) of the reconstructed time-resolved emission spectra (TRES).[1,6] It has been shown that the frequency of the $t = 0$ peak emission, $\nu(0)$, can be calculated quite accurately when the absorption and fluorescence spectra in a nonpolar reference solvent and the absorption spectrum in the system of interest are known.[6,11] If solvent relaxation completes during the lifetime of the excited state and the majority of the solvent relaxation process is captured with the given time resolution of the experiment, the fwhm's generally increase after the onset of excitation and reach a maximum at a time near to the average relaxation time, followed by a decrease.[1,6] When solvent relaxation is faster than the time resolution of the experiment, the rising edge is at least partially missing.[1,5] If solvent relaxation is slower than the fluorescence decay and thus cannot complete, only an increase of the fwhm is observed.[1,5]

Materials and Methods

All probes (2-(9-anthroyloxy)stearic acid (2-AS), 9-(9-anthroyloxy)stearic acid (9-AS), 11-((5-dimethylaminonaphthalene-1-sulfonyl)amino)undecanoic acid (Dauda), 6,8-difluoro-4-heptadecyl-7-hydroxycoumarin (C_{17}DiFU; at pH = 7.4 and incorporated in SUVs (small unilamellar vesicles) the compound is deprotonated), 6-hexadecanoyl-2-(((2-(trimethylammonium)ethyl)methyl)amino)naphthalene chloride (Patman), 6-propionyl-2-dimethylaminonaphthalene (Prodan), and 6-lauroyl-2-dimethylaminonaphthalene (Laurdan; see Figure 1 for structures)) were used as purchased from Molecular Probes. PC was supplied by Avanti Lipids. The preparation of PC SUVs was performed as described previously.[2−4] Fluorescence decays and spectra were recorded with a modified Edinburgh Instruments FSFL900 SPC equipment. Decay kinetics were recorded by using an IBH NanoLED-03 excitation source (370 nm peak wavelength, 1.2 ns pulse width, 1 MHz repetition rate) and a cooled Hamamatsu R3809U-50 microchannel plate photomultiplier attached to a Jobin-Yvon HR10 monochromator set to 8 nm resolution. The primary data consist of a set of emission decays recorded at a series of wavelengths spanning the steady-state emission spectrum. The fwhm's and emission maxima ($\nu(t)$) of the reconstructed TRES were obtained as described.[1−4,6] Absorption spectra have been recorded on a Perkin-Elmer Lambda 19 spectrometer. The time-zero spectrum and the corresponding $\nu(0)$ values were determined as described.[6,11]

Results and Discussion

Headgroup Labels Prodan, Laurdan, and Patman.[1−3,12,13] The time-dependent Stokes shift $\Delta\nu$ ($\Delta\nu = \nu(0) - \nu(\infty)$) depends on both the solvent polarity and the change in the solute's dipole moment. Since Prodan, Laurdan, and Patman contain practically identical chromophores, detected differences in $\Delta\nu$ (Table 1) directly reflect microenvironments of different polarity. It has been shown that the chromophore of Prodan is located in the

(10) Please note that the presented paper is exclusively dealing with solvent relaxation observed in the physiologically relevant liquid-crystalline phase. The solvent relaxation behavior in the gel phase of bilayers differs dramatically from the one reported within this work. Generally, the time-dependent Stokes shift $\Delta\nu$ in the gel phase is significantly smaller than when probed in the liquid-crystalline phase and the contribution of ultrafast solvent relaxation is much more pronounced than in the liquid-crystalline phase. See for example: (a) ref 5. (b) Bürsing, H.; Ouw, D.; Kundu, S.; Vöhringer, P. *Phys. Chem. Chem. Phys.* **2001**, *3*, 2378−2387. (c) Hutterer, R.; Hof, M. *Z. Phys. Chem.*, in press.

(11) Fee, R. S.; Maroncelli, M. *Chem. Phys.* **1994**, *183*, 235−247.

(12) Krasnowska, E. K.; Gratton, E.; Parasassi, T. *Biophys. J.* **1998**, *74*, 1984−1993.

(13) Parasassi, T.; De Stasio, A.; Ravagnan, G.; Rusch, R. M.; Gratton, E. *Biophys. J.* **1991**, *57*, 179−189.

Table 1. Characteristics of the Solvent Relaxation Process Probed by Seven Membrane Labels[a]

	$\Delta\nu$ (cm^{-1})[b]	τ_1 (ns)[c]	τ_2 (ns)[c]	τ_3 (ns)[c]	τ_r (ns)[d]	obsd SR (%)[e]
Dauda	3800	<0.2 (0.01)	<0.2 (0.06)	1.3	0.4	26
C_{17}DiFU	1700	<0.2 (0.04)	0.3	1.2	0.4	52
Prodan	3750	1.0			1.0	90
Laurdan	3450	1.7			1.7	86
Patman	3000	1.7			1.7	89
2-AS	2750	0.2	1.6		1.3	86
9-AS	2100	<0.2 (0.1)	0.7	4.0	1.6	77

[a] See Figure 1 for their localization in the lipid bilayer. [b] $\Delta\nu$ ($\Delta\nu = \nu(0) - \nu(\infty)$); $\nu(0)$ has been determined by time-zero spectrum estimation (refs 6 and 10), except for the deprotonated C_{17}DiFU. The $\nu(0)$ for deprotonated C_{17}DiFU has been estimated by recording the fluorescence emission spectrum in methanol/ethanol glass at 77 K. The values for $\nu(\infty)$ are obtained by TRES reconstruction (refs 1−4 and 6). [c] Solvent relaxation times resulting from (multi)exponential fitting of the $C(t)$ functions. The values in brackets are fit values, which are smaller than the given time resolution of the experiment. [d]

$$\langle\tau_r\rangle \equiv \int_0^\infty C(t)\,dt$$

[e] Obtained by comparison of the $\Delta\nu$ values determined by using the $\nu(0)$ values from the time-zero spectrum estimation (refs 6 and 10) with those obtained exclusively by TRES reconstruction.

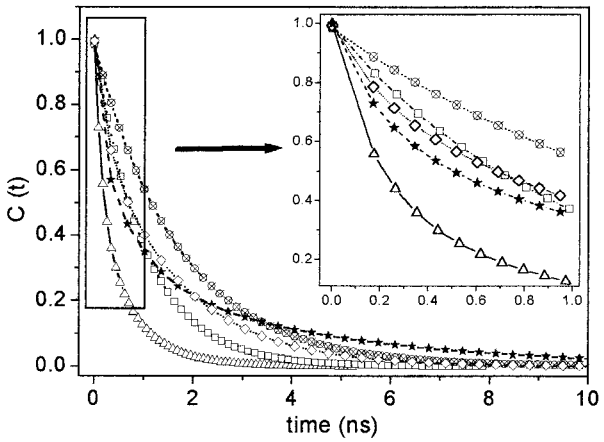

Figure 2. Correlation functions $C(t)$ for C_{17}DiFU (Δ), Prodan (□), Laurdan (⊗), 2-AS (◇), and 9-AS (★) in PC SUVs at ambient temperature. Shown are the (multi)exponential fits (see Table 1) to the experimental data, using $\nu(0)$ obtained by the time-zero spectrum estimation (refs 6 and 11). Since the $C(t)$ function for Patman is very similar to that obtained for Laurdan, it is not displayed. More than 70% of the solvent relaxation probed by Dauda is occurring on a shorter time scale than the experimental time resolution; the corresponding $C(t)$ is therefore not shown.

headgroup region of phospholipid bilayers close to the lipid/water interface.[1−3] Smaller $\Delta\nu$ values for Patman and Laurdan indicate that their chromophores are embedded deeper in the bilayer, which is in agreement with previous studies.[1−3,12,13] A comparison of the $\Delta\nu$ values determined by using the $\nu(0)$ values from the time-zero spectrum estimation[6,11,14] with those obtained exclusively by TRES reconstruction[1−4,6] shows that about 90% of the solvent relaxation probed by those three dyes is occurring on subnanosecond to nanosecond time scales (Table 1). This conclusion is confirmed by the time evolution of the fwhm (Figure 3). In all three cases, the fwhm's increase at early times and reach their maxima at 0.7 ns (Prodan) and 1.5 ns (Patman, Laurdan), followed by a decrease of the spectral width. The observed profiles prove that during the lifetime of the excited state solvent relaxation completes and almost the whole relaxation process is captured by an equipment providing subnanosecond time resolution. The correlation functions $C(t)$ ($C(t) = (\nu(t) - \nu(\infty))/\Delta\nu$) of all three headgroup labels can be fitted by a monoexponential function reasonably well (Figure 2 and Table 1). The resulting characteristic solvent relaxation time for Prodan is significantly smaller than those obtained for Patman and Laurdan. The comparison of the obtained data for these dyes gives evidence for a polarity and fluidity gradient within the PC bilayer headgroup. The results confirm the ability of these dyes to probe microfluidity and micropolarity changes within the headgroup region of biomembranes[1−3,5,12,13] using equipment providing subnanosecond resolution. Within the past years, several contributions appeared addressing the question on the origin of slow relaxation components in restricted media, as for example microemulsions, micelles, and lipids.[15,16] The authors propose that ionic headgroups can make the nearest water molecules nearly immobile and that those "bound" water molecules undergo slow exchange with "unbound" water molecules. Though such a dynamic exchange model sounds plausible, it considers only the role of the water molecules but not the direct influence of the charged headgroups on the solvent relaxation kinetics. The influence of the chemical structure of the phospholipid headgroups on the solvent relaxation monitored by Patman and Prodan has been demonstrated by our group.[3,5] In our opinion, a thorough understanding of the origin of the evident slow relaxation in phospholipid bilayers is not at all reached yet.

Phospholipid Backbone Labels 2-AS and 9-AS.[4] The *n*-AS probes ($n = 2, 6, 9, 12, 16$) are known to insert into the membrane with the stearoyl chains parallel to the phospholipid acyl chains. The long axis of the anthroyl ring is on average almost perpendicular to the plane of the membrane. The chromophore is positioned at a defined depth of the bilayer.[4] Thus, the anthroyl ring of 2-AS is located significantly closer to the membrane/water interface than that of 9-AS. If the chromophore (9-anthranoic ester) is located in a nonpolar environment, an intramolecular conformational change from the initially excited Franck−Condon state to the state with equilibrium geometry occurs. The formation of this relaxed geometry (characterized by more planar orientation of the aromatic ring relative to the carboxyl group) leads to a red-shift of the TRES with time after excitation.[4] On the other hand, in polar solvents the intermolecular, continuous solvent relaxation process governs the excited-state kinetics.[4] The $\Delta\nu$ value for 2-AS is significantly larger than that of 9-AS (Table 1), providing evidence for a polarity gradient within the hydrophobic backbone of the bilayer. Similar to the headgroup labels Prodan, Laurdan, and Patman, about 90% of the solvent relaxation probed by 2-AS occurs on the subnanosecond to nanosecond time scale. This conclusion is supported by the fwhm profile (data not shown in Figure 3) similar to the one observed for Prodan (Figure 3). In contrast to the examined headgroup labels, two exponentials are necessary to describe $C(t)$. The deviation from the monoexponentiality is even more pronounced for 9-AS. Visual inspection of both $C(t)$ functions (Figure 2) as well as the values listed in Table 1 support the view that a deeper localization of the anthranoic ester of 9-AS

(14) Inhomogeneity of probe localization might broaden the absorption spectra. The calculated frequency of the $t = 0$ peak emission, however, depends mainly on the maxima of the determined absorption spectra (ref 11), which appear to be rather unaffected by a possible inhomogeneity of probe localization.

(15) Bhattacharyya, K.; Bagchi, B. *J. Phys. Chem. A* **2000**, *104*, 10603−10613.

(16) Bhattacharyya, K.; Bagchi, B. *Chem. Rev.* **2000**, *100*, 2013−2045.

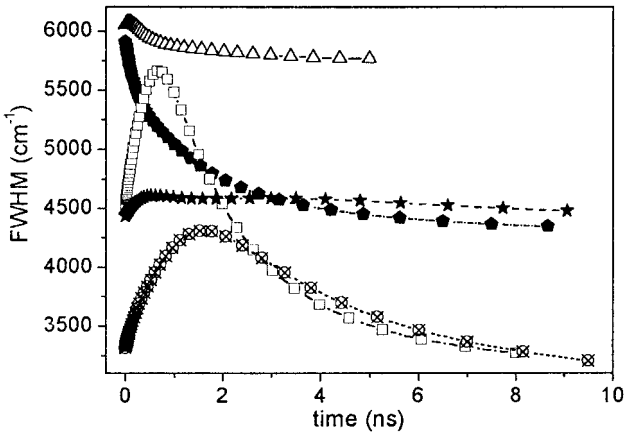

Figure 3. The fwhm of the TRES as a function of time after excitation for Dauda (⬟), C_{17}DiFU (△), Prodan (□), Laurdan (⊗), and 9-AS (★) in PC SUVs at ambient temperature. Since the fwhm time profiles obtained for 2-AS and Patman are very similar to those obtained for Prodan and Laurdan, respectively, they are not displayed.

leads to a larger amount of an "ultrafast"[17] red-shift of the TRES but to a slower solvent relaxation on the nanosecond time scale. We conclude for 2-AS that the transient red-shift of TRES[4] is predominately determined by solvent relaxation. Moreover, we can speculate that the ultrafast intramolecular relaxation process becomes more important when the chromophore is located at a larger distance from the lipid/water interface. The conclusion that besides solvent relaxation another relaxation process determines the red-shift of the TRES obtained for 9-AS is supported by its fwhm profile which is rather untypical for pure solvent relaxation (Figure 3). A more detailed study on this issue is in preparation.

(17) In the context of this work, we mean by the term "ultrafast" processes which are not resolvable with the time resolution of the experiment (~200 ps).

Dauda and C_{17}DiFU Probing the External Interface of the Bilayer.[18,19] Inspection of the fwhm (Figure 3) and estimation of the ultrafast solvent relaxation contribution (Table 1) indicate that a substantial part of the solvent relaxation process probed by Dauda and C_{17}DiFU has not been captured by the experiment. Only 26% of the solvent relaxation probed by the dansyl chromophore of Dauda occurs on the subnanosecond time scale. Bearing in mind the known tendency of dansyl to loop up toward the water phase,[18] the obtained solvent relaxation kinetics and fwhm profile lead to the conclusion that Dauda mainly probes the properties of water within the external interface of the bilayer. It has been shown that the neutral coumarin chromophore of 4-heptadecyl-7-hydroxycoumarin is located about 0.2 nm from the bilayer surface.[19] The kinetic data and the estimated amount of ultrafast solvent relaxation contribution (Table 1 and Figure 2) reveal that the anionic 6,8-difluoro-7-hydroxycoumarin chromophore of C_{17}DiFU is possibly localized within the external interface of the bilayer, too. A comparison with Dauda indicates a somewhat closer localization of the coumarin chromophore toward the headgroup region of the bilayer when compared with the dansyl chromophore.

Acknowledgment. We thank the Ministry of Education, Youth and Sports of the Czech Republic (via LN 00A032) and the Grant Agency of the Czech Republic (Grant No. 203/99/0845) for financial support. We thank Mark Maroncelli, Arno Münster, and Rudi Hutterer for providing computer programs for the time-zero spectrum estimation, installing those programs, and critically reading the manuscript, respectively.

LA011337X

(18) Asuncion-Punzalan, E.; Kachel, K.; London, E. *Biochemistry* **1998**, *37*, 4603−4611.
(19) Kraayenhof, R.; Sterk, G. J.; Sang, H. W. W. F. *Biochemistry* **1993**, *32*, 10057−10066.