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TRANES Spectra of Fluorescence Probes in Lipid Bilayer Membranes: An Assessment of Population Heterogeneity and Dynamics

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Time-resolved fluorescence of eight fluorescence probes were studied in EggPC bilayer membrane vesicles. Emission wavelength dependent fluorescence decays were analyzed in a model-independent way to obtain time resolved area normalized emission spectra (TRANES). The TRANES spectra of the probes studied were classified into four types: (1) spectra that are identical at all time (one emissive species), (2) spectra that show an isoemissive point (two emissive species), (3) spectra that shift continuously with time (slow solvation dynamics or multiple species), and (4) spectra that shift for a short time and thereafter one or two emissive species are indicated. The TRANES spectra of these eight probes, except RH421, belong to the type 1, 2, or 4. The continuous shift of the TRANES spectra that was observed for the probe RH421 is attributed to multiple ground state species and not due to slow solvation dynamics.

KEY WORDS: Fluorescence spectroscopy; TRES; TRANES; bilayer membranes; solvation dynamics; heterogeneity.

INTRODUCTION

Time resolved fluorescence studies have proven to be extremely useful for understanding the structure and dynamics in complex systems such as biological and model membranes using intrinsic or extrinsic fluorescence probes [1]. In complex biological and biomimetic systems the time resolved fluorescence decay is often multiexponential and emission wavelength dependent. Emission wavelength dependent fluorescence decays are used to construct time-resolved emission spectra (TRES) of fluorescence probes, which are useful to obtain information on the excited state kinetics and heterogeneity of emissive species in a system [2,3]. It is important to know the number of emissive species for the interpretation of fluorescence results, including TRES in complex systems. Recently, time-resolved area-normalized emission spectroscopy (TRANES), a model-independent method, was proposed for the analysis of emission wavelength dependent fluorescence decays [4–6]. TRANES spectra are obtained by a simple extension to TRES by normalizing TRES spectra to have a constant area. The main advantage of TRANES spectra is their interpretation without recourse to any model for determining the number of emissive species in the sample. For example, emission from a single species is indicated by identical TRANES spectra. Observation of an isoemissive point in TRANES spectra is an unambiguous confirmation for the presence of two emissive species in the sample, irrespective of their origin [4]. Observation of two isoemissive points at different times indicates three emissive species [6], and so on.

Fluorescence of organic dye molecular probes is widely used in the investigation of the physical properties

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of the lipid membranes in model and biological systems [7–9]. The structure and dynamics of the probe molecule bound to the membrane are of great interest because of the usefulness of their fluorescence properties in a wide variety of biological applications [10,11]. One or more fluorescence properties (e.g., spectra, quantum yield, lifetime, anisotropy, etc.) of the molecule are sensitive to variation in the structure and/or dynamics of the membrane. The exact molecular mechanism for the change in fluorescence property in some applications (e.g., the action of dyes that are sensitive to the membrane potential [12–14]) is not clearly understood. Identification of the dye populations present within the lipid membrane would be helpful in understanding the molecular mechanism underlying the change in fluorescence properties.

In many complex biological and biomembrane systems it is likely that there are more than two emissive species. For example, a fluorescence probe may be distributed between aqueous phase and membrane phase. In the membrane phase itself, the probe may be distributed between various locations such as surface and interior regions [15]. In the lipid membrane an organic molecule can be located in three distinct regions: (i) The surface region where the dye is exposed to the external aqueous phase, (ii) the interface region where the dye exposure to the aqueous phase is limited, and (iii) the core region where the dye is entirely in a hydrophobic environment. Presence of the dye in more than one location constitutes a multicomponent system (ground state heterogeneity) even though a single dye is used as a probe.

Another important situation in lipid membranes is the solvent relaxation or solvation dynamics reported by the emissive species during its lifetime. Solvation dynamics is a useful indicator of the physical property such as viscosity and polarity of the local environment of the dye. In liquid solutions, the dye probe exists as a single species in the ground state and the time scale of solvation dynamics is in the order of picoseconds. In membranes and other constrained media, solvation dynamics was reported to be in nanoseconds, which is usually slow [16,17]. The conclusions on solvation dynamics in heterogeneous media were based on the interpretation of TRES spectra assuming that there is only one species in the ground state and emission originates from a continuum of energy levels in the excited state. Analysis of TRANES spectra is essential to conclude that solvation dynamics does indeed occur in constrained media.

In this paper, we have used the method of TRANES to investigate the nature of the fluorescence emission of several fluorescence probes in lipid bilayer membranes and examined the relevance of solvation dynamics in these cases.

EXPERIMENTAL

The dyes used in this study are DODCI (3,3′-diethyloxacarbocyanine iodide), BODIPY 611/627 (3,5-bis-(2-thienyl-4,4-difluoro-4-bora)-3a,4a-diaza-s-indacene), Merocyanine540 (MC540), DPH (1,6-diphenyl-1,3,5-hexatriene), RH421 [*N*-(4-sulfobutyl)-4-(4-(4-dipentylamino)phenyl)butadienyl)pyridinium, inner salt] (all from Molecular Probes, USA) and $DiSC_3(5)$ (3,3′-dipropylthiadicarbocyanine iodide), Rhodamine 6G, and Nile red (from Sigma-Aldrich Chemicals, USA).

Steady state fluorescence spectra were recorded using a spectrofluorimeter (SPEX Fluorolog FL111 T-format) and corrected for the spectral sensitivity of the photomultiplier (Hamamatsu R928A). Time-resolved fluorescence measurements were carried out by time-correlated single-photon counting (TCSPC) method. A CW mode-locked frequency-doubled Nd:YAG laser (Spectra Physics) driven dye (Rhodamine 6G) laser, which generates 4–10 ps pulses at 800 kHz was used as the excitation source for samples that were excited at 571 nm/575 nm. A CW mode Nd:Vanadate (Millenia, Spectra Physics, USA) pumped mode-locked Ti-Sapphire laser, double/tripled tunable output for exciting samples at 460 nm or 307 nm. The fluorescence emission at magic angle (54.7°C) was dispersed in a monochromator (fl, spectral width 2.5 nm) and counted ($3–4 \times 10^3$ s^{-1}) by a MCP PMT (R2809), and processed through CFD, TAC, and MCA. The instrument response function was ~40 ps.

Time resolved fluorescence measurements were recorded for the dyes in EggPC (or EggPC + 30% (w/w) cholesterol) over the entire emission spectrum at 5-nm intervals except in the wings where the interval was 10 nm. The EggPC vesicles were prepared by sonication method. In the sample, the lipid concentration was 4 mg/ml and dye/lipid ratio was 1:300. The buffer in all the cases was 150 mM KCl and 20 mM KH_2PO_4, pH 7.5.

Data Analysis

The TRANES spectra of various dyes in EggPC vesicles were obtained in four steps as follows [4].

Step 1

Fluorescence decays of probes were obtained in the entire emission spectrum of the probe at 5-nm intervals. The peak count in the fluorescence decay was 1×10^4 for all wavelengths except in the wings of the spectrum for which the peak count was 5×10^3.

Step 2

Fluorescence decay at each wavelength was deconvoluted using the instrument response function and a multiexponential function, $I(t) = \Sigma\alpha_i \exp(-t/\tau_i)$, $i = 1–4$, where α_i can be negative (for excited state kinetics) by the standard method of nonlinear least squares and iterative reconvolution. A four exponential function was found to be adequate for all the decays. The sole aim of this step is to obtain a noise-free representation of the intensity decay function (for a hypothetical δ-function excitation), $I(t)$, at each wavelength. Hence, the amplitudes and lifetimes obtained in this fit do not have any physical meaning.

Step 3

Time resolved emission spectra (TRES), plotted as intensity vs. wavelength, were constructed using $\alpha_i(\lambda)$ and $\tau_i(\lambda)$, and steady-state emission spectrum corrected for the quantum efficiency of the photomultiplier. The equation used is,

$$I(\lambda,t) = I_{SS}(\lambda)\frac{\sum_j \alpha_j(\lambda)e^{\frac{-t}{\tau_j(\lambda)}}}{\sum_j \alpha_j(\lambda)\tau_j(\lambda)}$$

where, $I_{ss}(\lambda)$ is the steady state fluorescence intensity at λ, $\alpha_j(\lambda)$ and $\tau_j(\lambda)$ are the values of fit parameters.

Step 4

TRANES spectra were constructed by normalizing the area of each spectrum in TRES such that the area of the spectrum at any time t is equal to the area of the spectrum at $t = 0$.

RESULTS AND DISCUSSION

TRES and TRANES spectra for eight fluorescence dyes in EggPC membrane were obtained as described in the experimental section. These spectra showed four different trends. (1) The TRES spectra are unchanged with time and TRANES spectra are identical at all time. (2) TRES spectra change with time and an isoemissive point is observed in the TRANES spectra, (3) The TRES and TRANES spectra shift continuously with time, and (4) The TRES and TRANES spectra shift for $t < t_x$ and thereafter the spectra are identical or an isoemissive point is observed. The steady state spectrum is different from the TRANES spectra in all cases except the first one. The above trends for TRES and TRANES indicate different interpretation of the fluorescence behavior of the dyes in the membrane and hence the results are described and discussed in the above order.

1. TRANES Spectra Are Identical

The peak position and spectral shape of TRES spectra are unchanged with time. The spectra are identical to the steady state spectrum. This is the simplest case. This indicates that the emission occurs from a single species. The TRANES spectra for DPH in the membrane showed no change with time (Fig. 1). TRES spectra (also shown in Fig. 1) are similar to TRANES spectra except that the intensity was diminishing with time. The inset in the figure shows that the peak of the spectrum does not shift with time. The dye is present as a spectroscopically distinct population localized in one type of environment, namely, the core region of the lipid bilayer.

Interestingly, the fluorescence decay of DPH in bilayer membrane is not a single exponential. It is reported to be multiexponential with two or three lifetimes [7,15,18]. The decay was found to be a two-exponential function in the present study also. The observed lifetimes are 8.35 and 3.5 ns, and the amplitudes are 0.7 and 0.3, respectively. The observation of multiexponential decay may appear to contradict the conclusion that the emission is from a single species based on TRANES spectra. This apparent contradiction is resolved as follows. Recent experiments have shown that the radiative rate of DPH in bilayer membrane depends on the orientation of the dye [15,18] with respect to the membrane normal. The effect of refractive index on the radiative rate of DPH in membrane was used to identify two orientational distributions for DPH in the membrane [19]. It can be argued therefore that DPH in the core region has the same emission spectrum but differ in their radiative rate because of orientation. Hence, the observation of multiple fluorescence lifetimes for a single emissive species of DPH in membrane is justified.

2. TRES Spectra Shift with Time but an Isoemissive Point Is Observed in the TRANES Spectra

This is a straightforward case indicating that there are only two emissive species in the sample. It was shown by mathematical analysis that an isoemissive point in the TRANES spectra is the necessary and sufficient condition for the presence of two emissive species irrespective of their origin [4]. Examples of different cases that involve two emissive species are previously discussed [4]. There are two possibilities that give rise to two emissive species. In the first one, the dye exists as a single population in

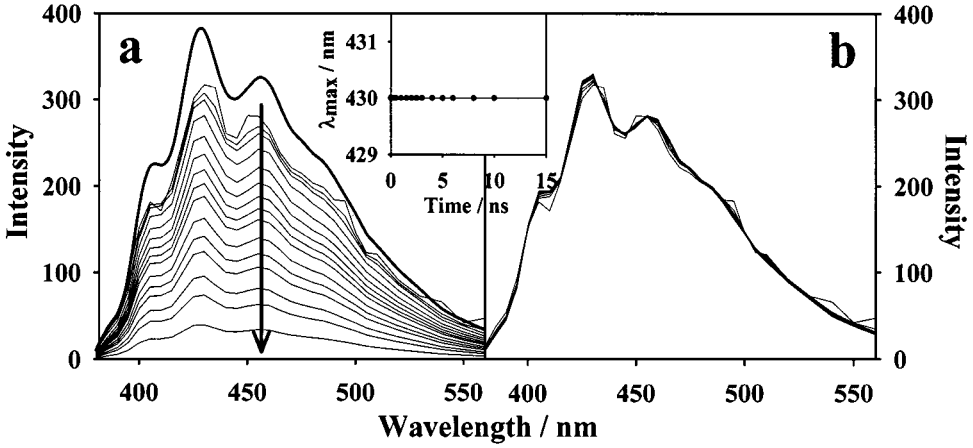

Fig. 1. DPH (15 μM) in EggPC membrane. λ_{ex} = 307 nm. (a) TRES and (b) TRANES spectra at 0, 0.1, 0.2, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, and 15 ns. Direction of the arrow indicates from 0 to 15 ns. Thick line is the steady state fluorescence emission spectrum. (Inset) Shows the peak maximum in TRES and TRANES with time.

the ground state and two emissive species are formed by excited state kinetics. The second possibility is that the dye is present as two spectroscopically distinct species in the ground state and both are excited to two distinct emissive species which may or may not be kinetically coupled. These two possibilities can be distinguished by examining the nature of the fluorescence decays at all emission wavelengths. In the first case, one must find evidence for a decay component with negative amplitude at long emission wavelengths. In the second case, the fluorescence decay must not have a lifetime component with negative amplitude. In both cases, the peak of TRES spectra may appear to shift with time if the spectral maximum of the two species are different. The distinction may not be possible if ultrafast kinetics and/or extensive overlap of spectra of the two species are present.

The TRANES spectra for Nile red and $DiSC_3(5)$ showed an isoemissive point (Figs. 2 and 3). In the case of Nile red, only one isoemissive point is observed at 625 nm whereas in the case of $DiSC_3(5)$ two isoemissive points at 687 and 718 nm were observed. The peak of the spectrum shifts with time for Nile red whereas the peak position does not change for $DiSC_3(5)$. The analysis fluorescence decays indicated that a lifetime component with negative amplitude was not found at any emission wavelength. This indicates the absence of excited state kinetics. We conclude that there are two populations of the dye in the ground state and these give rise to two emissive species. Observation of two isoemissive points in the case of $DiSC_3(5)$ may be explained as follows: The two species have the same emission maximum but the spectrum is broad for one species and narrow for the second species. This will give rise to the two isoemissive points as observed.

TRES and TRANES Spectra Shift Continuously with Time

There are two possible mechanisms that give rise to TRES and TRANES spectra that shift continuously with time. In the first case, the excited state of a single species undergoes solvation dynamics in a time scale that is comparable to the fluorescence decay time. An example of this is solvation dynamics of polar dye molecules in viscous polar solvents [4]. In this case, the emission occurs from a continuum of energy levels that are in between the initial excited state and the final relaxed species and the relaxation process may not be completed during the excited state lifetime. In the second case, the dye is extensively heterogeneous in the ground state and as a result the excited state is also extensively heterogeneous. The two cases may be distinguished by examining the nature of the fluorescence decay. The former case of solvation dynamics must be confirmed by a lifetime component with negative amplitude especially at longer emission wavelengths. In the latter case, the decay may not have any lifetime component with negative amplitude.

The probe RH421 showed a continuous red shift in the spectra (Fig. 4). A continuous red shift in TRANES spectra could mean either pure solvation dynamics of a single species or extensive heterogeneity in the ground state. The absence of a lifetime component with negative pre-exponential factor at the longer emission wavelengths rules out solvent relaxation and in favor of multiple spe-

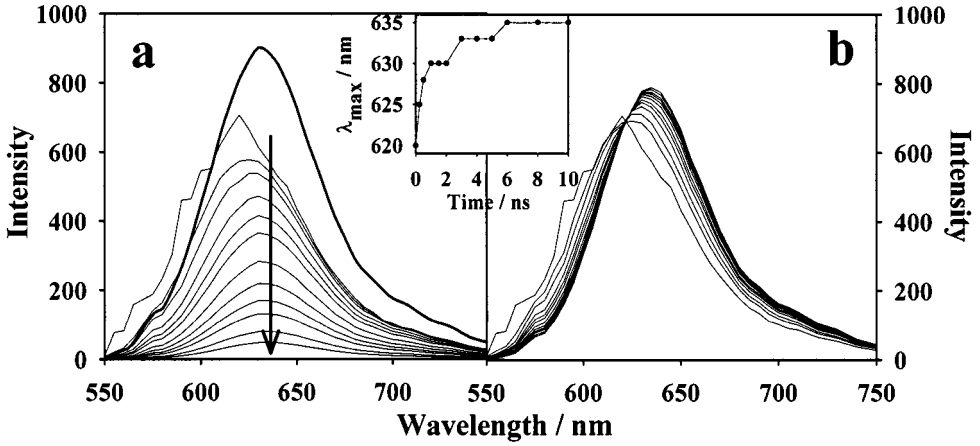

Fig. 2. Nile red (15 μM) in EggPC membrane. λ_{ex} = 571 nm. (a) TRES and (b) TRANES spectra at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, and 10 ns. Direction of the arrow indicates from 0 to 10 ns. Thick line is the steady state fluorescence emission spectrum. (Inset) Shows the peak maximum in TRES and TRANES with time.

cies in the ground state [20]. This case is further discussed in a later section.

TRES and TRANES Spectra Shift Continuously for $t < t_x$ ps, and Thereafter the Spectra Are Either Identical or an Isoemissive Point Is Observed

Based on the above results and discussions one may conclude that in this case the emission is from a single species (identical TRANES spectra) or two species (isoemissive point) but only after t_x. For $t < t_x$, one may consider that a relaxation process occurs. This relaxation may be due to solvation dynamics or a short-lived species with a lifetime less than t_x. The validity of either mechanism will have to be confirmed by experimental data obtained with high accuracy in $t < t_x$ time scale.

The TRANES spectra for MC540 in EggPC membrane showed that the spectral maximum shifts for $t <$ 50 ps and thereafter the spectra are identical (results not shown). The TRANES spectra for DODCI, Rhodamine 6G and BODIPY showed a spectral shift for $t <$ 100 ps and thereafter an isoemissive point was observed (results not shown). Experimental data with accuracy better than that used in this study are required to interpret the spectral shift in TRANES in sub–100 ps time scale. Therefore, further analysis of TRANES spectra of these cases was not attempted.

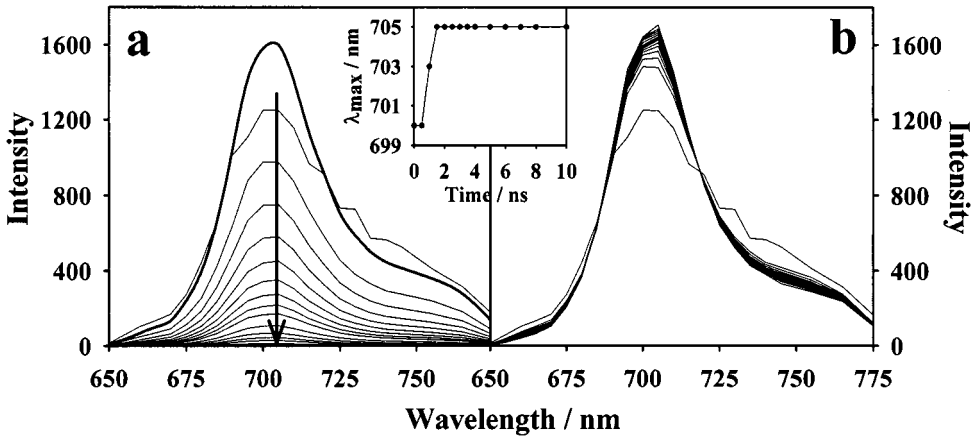

Fig. 3. $DiSC_3(5)$ (15 μM) in EggPC membrane. λ_{ex} = 624 nm. (a) TRES and (b) TRANES spectra at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, and 10 ns. Direction of the arrow indicates from 0 to 10 ns. Thick line is the steady state fluorescence emission spectrum. (Inset) Shows the peak maximum in TRES and TRANES with time.

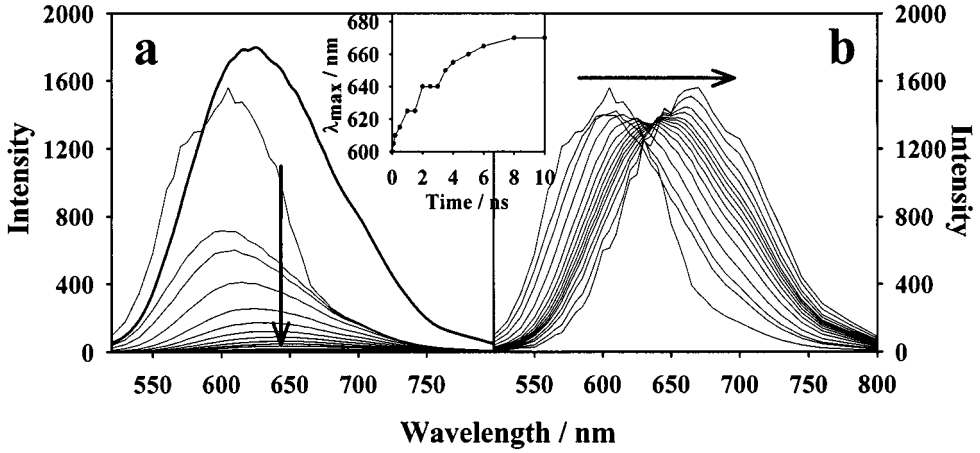

Fig. 4. RH421 (15 μM) in EggPC membrane. λ_{ex} = 460 nm. (a) TRES and (b) TRANES spectra at 0, 0.1, 0.2, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, and 10 ns. Direction of the arrow indicates from 0 to 10 ns. Thick line is the steady state fluorescence emission spectrum. (Inset) Shows the peak maximum in TRES and TRANES with time.

3. TRANES Spectra of Nile Red in EggPC Membrane with Cholesterol

The results and discussion based on TRANES spectra of fluorescent dyes in bilayer membrane indicated marked differences in the fluorescence characteristics of the dyes. Fluorescence of DPH is due to a single species, that of Nile red is due to two emissive species, and that of RH421 is due to multiple species. Other dyes indicated that there might also be a fast component with a time constant of 50–100 ps, which is followed by emission from a single or two species. We have chosen one dye, Nile red, to investigate its fluorescence characteristics by TRANES when the state of membrane is altered by the addition of cholesterol.

Nile red is a membrane probe that is highly sensitive to the polarity of its environment [21]. An earlier study in solvents of different polarity revealed that the fluorescence lifetime of Nile red increased with a decrease in solvent polarity [21]. A lower lifetime is therefore, indicative of higher polarity in the environment. As described earlier, TRANES spectra of Nile red in EggPC showed an isoemissive point at 625 nm (Fig. 2) which indicates that two emissive species are present in the sample. There was no fast relaxation or a lifetime component with negative amplitude. This implies that in EggPC membrane, Nile red exists as two populations in the ground state. The TRANES spectra were obtained for Nile red in EggPC membrane containing cholesterol (30% w/w) and the spectra are shown in Figure 5. The spectra showed an isoemissive point at 615 nm, which is blue-shifted, compared to 625 nm for the dye in the membrane without cholesterol. There was no fast relaxation component or a lifetime component with negative amplitude at any wavelength. The spectrum is however broad in the presence of cholesterol. We conclude that the presence of cholesterol in the membrane alters the environment significantly thereby making spectrum of the dye a broad one.

Observation of the isoemissive point with and without cholesterol suggests that the dye is distributed essentially as two populations in the membrane. Nile red being a hydrophobic molecule, it is expected to localize in the interior hydrophobic region and interface region of the bilayer [15]. Addition of cholesterol appears to change the local environment and hence the spectral characteristics of both these populations. Our previous study of fluorescence lifetime distribution of Nile red using maximum entropy method (MEM) had shown that there is an increase in the heterogeneity with the addition of cholesterol to EggPC membrane [22]. This was evident from the increased width of the lifetime distribution in cholesterol containing membrane. Cholesterol is known to reduce the water content of membranes [23–28] as seen by the longer lifetime of Nile red in the cholesterol containing membranes. The blue shifted isoemissive point and a broad emission spectrum in the cholesterol containing membranes may also be due to the lowered water content in these membranes. The results and analysis of TRANES spectra are therefore in good agreement with the conclusions based on MEM analysis for the fluorescence decay of Nile red in lipid membranes with and without cholesterol. Further, by reporting that the number of emissive species in the sample is two, they add strength to the conclusion based MEM analysis of fluorescence decays [22].

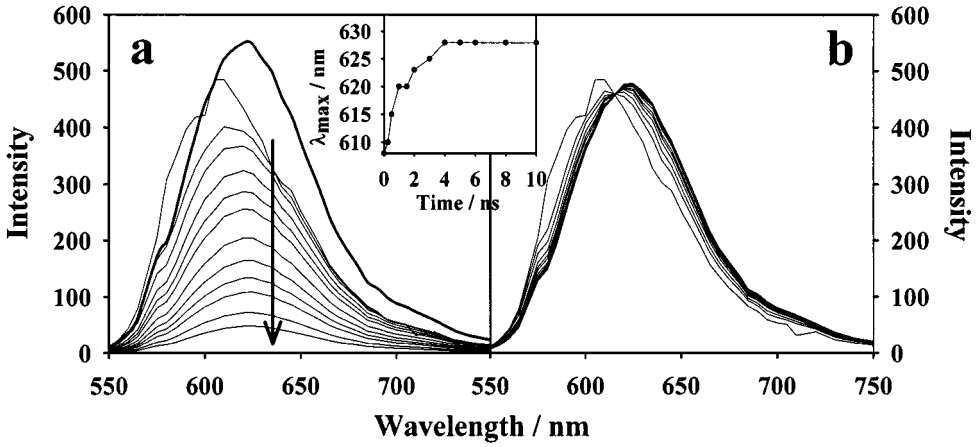

Fig. 5. Nile red (15 μM) in EggPC membrane + 30% cholesterol. λ_{ex} = 571 nm. (a) TRES and (b) TRANES spectra at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, and 10 ns. Direction of the arrow indicates from 0 to 10 ns. Thick line is the steady state fluorescence emission spectrum. (Inset) inset shows the peak maximum in TRES and TRANES with time.

4. Solvation Dynamics of Dyes in Bilayer Membranes

Fluorescence probes are often used to investigate the "solvent" characteristics in the interfacial or core region of the bilayer [16] and other similar organized molecular assemblies [17]. Here, solvent refers to either the water molecules that are tightly bound or penetrated into the membrane. Solvation dynamics occurs because the dipole moment or its direction of the fluorescent molecule in the excited state is different from that in the ground state. It is the process of energy relaxation of the excited state due to solvent/solute reorientation to adjust to the new value or direction of the dipole moment of the excited state. In principle, there is a continuum of energy levels from the initially excited state to the final relaxed one. The existence of a continuum of energy levels is observed in the solvation dynamics of dye molecules particularly in polar organic liquids [29]. In these studies, shift of the peak of time resolved emission spectra (TRES) were used for the quantitative measure of solvation parameters. The time scale of solvation depends on the viscosity and it varies from sub-picosecond to a few tens of picoseconds in most organic liquids. The same strategy based on experimental TRES spectra and associated mathematical analysis were used to study the solvation dynamics of probes in membranes and in other microheterogeneous structures [16,17]. That is, a continuous red shift with time observed in the TRES spectra of a dye in membrane was interpreted to indicate solvation dynamics. In membranes, the possibility exists that there are more than one ground state species and fluorescence emission is a sum of spectra of different species. Therefore, the interpretation of red shift in TRES spectra requires further careful analysis.

In liquids, the dye is present as a single species in the ground state. In bilayer membranes, there are at least three sites of solubilization for the dye: the aqueous surface region, the interface region and the core region. The physical properties of these regions differ very substantially. The fluorescence of several dyes solubilized in the membrane indicates that the probe is solubilized in at least two regions [19]. Thus, ground state heterogeneity is likely to be a common occurrence for most dyes when they are solubilized in membranes. If this were so, then the observed TRES spectra would be a sum of individual TRES spectra of two or more species. Nile red in EggPC with and without cholesterol is a good example that shows a continuous red shift in TRES for 4 to 6 ns (Figs. 2 and 5). TRANES analysis showed that emission occurs from two species whose spectral maxima are close to each other. Thus, the red shift with time in TRES spectra cannot be attributed to solvation dynamics of a single species.

It is shown that by extending the spectral analysis from TRES to TRANES one can unambiguously distinguish between a true continuum of energy levels and a sum of spectra two species [4]. Evidence that the molecule is present as a single species in the ground state and a continuous time-dependent red shift in TRES *and* TRANES spectra are the important requirements for the solvation dynamics model. Proof of existence of a single species in the ground state may be obtained in two ways. First, the excitation spectrum must be independent of the emission wavelength. Second, one must have kinetic evidence that the fluorescence decay at long emission

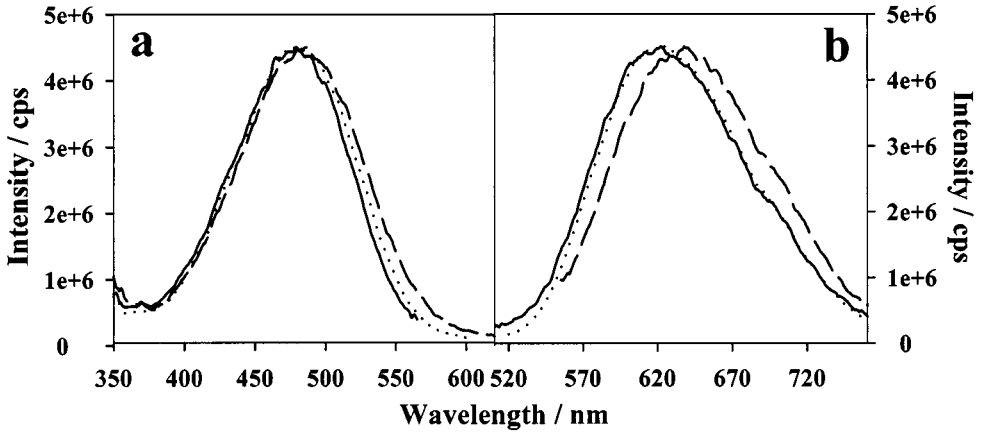

Fig. 6. Fluorescence spectra of RH421 (15 μM) in EggPC membrane. (a) Fluorescence excitation spectra at λ_{em} = 580 nm (thick line), λ_{em} = 620 nm (dotted line) and λ_{em} = 680 nm (dashed line). (b) Fluorescence emission spectra at λ_{ex} = 420 nm (thick line), λ_{ex} = 480 nm (dotted line) and λ_{ex} = 540 nm (dashed line).

wavelengths has a growth component (that is, a lifetime component with negative amplitude).

We now examine the relevance of solvation dynamics of the fluorescence probes in EggPC membranes used in this study. TRES and TRANES spectra of DPH did not show any spectral shift with time. There is no solvation dynamics in this case. TRES and TRANES spectra of $DiSC_3(5)$ did not show any spectral shift with time and an isoemissive point in TRANES spectra. Nile red (with and without cholesterol) showed time dependent spectral shift in TRES but an isoemissive point in TRANES. An isoemissive point is an unambiguous indication for two emissive species and not a continuum of species. Therefore, solvation dynamics is absent even though the peak of TRES spectra shift with time.

A continuous time-dependent red shift in TRES as well as TRANES was observed only for RH421 in EggPC membrane. Inset in Figure 4 shows the time dependence of the spectral maximum of TRES and TRANES spectra with time. Unfortunately, this is still not enough to invoke the solvation dynamics model. It is also necessary to establish that the dye exists as a single species in the ground state and a growth kinetics must be observed in the fluorescence decays at longer emission wavelengths. In the case of RH421 in EggPC membrane one must observe growth kinetics in the emission wavelengths $>$700 nm (Fig. 4a). The analysis of the decays in these wavelengths did not indicate a lifetime component with a negative amplitude. Growth kinetics with a solvent relaxation time of 50 ps or greater would have been detected unambiguously. Thus, the solvation dynamics model may be ruled out as the explanation for time-dependent red shift. The alternative interpretation that the ground state of RH421 is extensively heterogeneous holds well. This conclusion is also supported by the shift of emission spectrum with the excitation wavelength and vice versa (Fig. 6).

Red shift of emission spectrum of fluorescent dyes when excited at the red-edge of the absorption spectrum is commonly observed for polar dyes in bilayer membranes [30]. There were extensive studies on the red-edge effects of fluorescent dyes in bilayer membranes, microheterogeneous media and viscous polar solvents and the progress has been reviewed recently [31]. Red-edge effect is generally attributed to a heterogeneity of dye in the ground state and slow solvation dynamics. Unambiguous assignment of red-edge effect to solvation dynamics would require the time scale of solvation dynamics to be slow when compared to fluorescence lifetime. Distinction between ground state heterogeneity and solvation dynamics may be more unambiguous if one uses a fluorescent molecule that is located in a specific site in the membrane, for example, by covalently linking it to the lipid. Fluorophores localized in specific sites in membranes are therefore ideal cases for investigating slow solvation dynamics or solvent relaxation in the excited state by TRANES analysis.

CONCLUSIONS

Fluorescence behaviour of organic probe molecules in lipid bilayer membranes is considerably complex (e.g., multiexponential decay, wavelength dependent fluorescence, etc) and may exhibit considerable variations from probe to probe. The complexity arises due to multiple sites for the dye in the membrane (e.g., core, surface and interface regions), ground and excited state interactions

with the lipid/water that may differ in different sites, effect of refractive index and viscosity on the radiative and nonradiative rates of the dye that depend upon the local structure and physical properties. TRANES may be used to determine the number of emissive species in a model-independent way. Our investigations for several commonly used fluorescence probes in EggPC bilayer membrane vesicles have shown TRANES spectra that can be classified into four different types of behavior. Emission from a single species (e.g., DPH), two species (Nile red) and multiple species (RH421) were identified. Our analyses, by TRANES, have further shown that time-dependent changes in TRES cannot be taken as an indication of solvation dynamics in complex systems such as membranes.

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