Effect of Urea on the Organization and Dynamics of Triton X-100 Micelles: A Fluorescence Approach

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Received: August 21, 2003; In Final Form: December 4, 2003

Chaotropes such as urea perturb the organization of molecular assemblies formed by the hydrophobic effect. We have monitored the change in the organization and dynamics of Triton X-100 micelles induced by urea utilizing wavelength-selective fluorescence and related approaches using the environment-sensitive fluorescent membrane probes NBD-PE (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine) and DPH (1,6-diphenyl-1,3,5-hexatriene). NBD-PE is a phospholipid in which the headgroup is covalently attached with the fluorescent NBD group. On the other hand, DPH is an extensively used fluorescent membrane probe which partitions into deeper hydrocarbon region of membranes and micelles. Our results utilizing several sensitive fluorescence parameters (such as emission maximum, anisotropy, lifetime, and quenching) show increased polarity around the NBD group of micelle-bound NBD-PE in the presence of varying concentrations of urea. This could possibly be due to increased water penetration in Triton X-100 micelles induced by urea. Data from experiments using the deeper hydrocarbon region probe DPH support this observation. Interestingly, the extent of red edge excitation shift (REES) was invariant in the presence of varying concentrations of urea. Fluorescence quenching measurements of the micelle-bound NBD-PE using the aqueous quencher Co^{2+} indicate increased accessibility of the NBD group at higher urea concentrations. These results could be due to an increase in solvation of the polar headgroups by urea−water mixture than water alone, along with an increase in water penetration.

Introduction

Detergents are extremely important in studies of biological membranes because of their ability to solubilize membrane proteins.[1−3] They are soluble amphiphiles and above a critical concentration (strictly speaking, a narrow concentration range), known as the critical micelle concentration (CMC), self-associate to form thermodynamically stable, noncovalent aggregates called micelles.[4] The studies on micellar organization and dynamics assume special significance in light of the fact that the general principle underlying the formation of micelles (that is, the hydrophobic effect) is common to other related assemblies such as reverse micelles, bilayers, liposomes, and biological membranes.[4−7] Micelles are highly cooperative, organized molecular assemblies of amphiphiles and are dynamic in nature.[8,9] Further, they offer certain inherent advantages in fluorescence studies over membranes since micelles are smaller and optically transparent, have well-defined sizes, and are relatively scatter-free.

In general, chaotropes such as urea perturb the organization of molecular assemblies whose formation is driven by the hydrophobic effect.[10,11] This is due to their ability to weaken hydrophobic forces in aqueous solutions. It is therefore expected that chemical agents such as urea will influence the organization and stability of assemblies such as micelles which are determined by the delicate balance between hydrophilic and hydrophobic forces. It has been well established that urea, a commonly used protein denaturant, affects the formation and structures of nonionic micelles such as Triton X-100.[12,13] Triton X-100 is a mild nonionic (uncharged) detergent containing polyoxyethylene chains as hydrophilic moieties characterized by a low CMC. It is widely used in membrane biochemistry for solubilization of membrane proteins in the functional state.[2] Triton X-100 has been extensively used in the past few years to explore the detergent resistance (insolubility) of membrane fractions which could be indicative of the presence of membrane domains.[14] In addition, Triton X-100 has been used as a substrate diluent for enzymes such as phospholipases and lipases.[15] Interestingly, it has recently been shown that the activity of lipases increases in Triton X-100 micelles in the presence of chaotropes.[16] The organization and dynamics of Triton X-100 micelles in the presence of urea therefore assumes significance.

A direct consequence of any organized molecular assembly (such as micelles) is the restriction imposed on the dynamics and mobility of the constituent structural units. We have previously shown that the microenvironment of molecules bound to such organized assemblies can be conveniently monitored using the wavelength-selective fluorescence approach.[17,18] Wavelength-selective fluorescence comprises a set of approaches on the basis of the red edge effect in fluorescence spectroscopy which can be used to directly monitor the environment and dynamics around a fluorophore in an organized molecular assembly.[19−22] A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of absorption band, is termed red edge excitation shift (REES).[19−24] This effect is mostly observed with polar fluorophores in motionally restricted environments such as viscous solutions or condensed phases where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime.[19−26] REES arises because of slow rates of solvent relaxation (reorientation) around an excited state fluorophore, which is dependent on the motional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore. Utilizing this approach, it becomes possible to

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10.1021/jp0365007 CCC: $27.50
Published on Web 01/22/2004

probe the mobility parameters of the environment itself (which is represented by the relaxing solvent molecules) using the fluorophore merely as a reporter group. This makes the use of REES in particular and the wavelength-selective fluorescence approach in general very useful since hydration plays a crucial modulatory role in the formation and maintenance of organized molecular assemblies such as micelles, membranes, and folded proteins in aqueous solutions.[27]

We have previously shown that REES and related techniques (wavelength-selective fluorescence approach) serve as powerful tools to monitor organization and dynamics of probes and peptides bound to membranes,[28−37] membrane-mimetic media such as micelles,[17,18] and reverse micelles.[38,39] In addition, we have previously used the wavelength-selective fluorescence approach to analyze the organization and dynamics of tryptophans in the soluble hemolytic protein α-toxin[35] and the cytoskeletal proteins tubulin[40] and spectrin.[41]

In this report, we have monitored the change in the organization and dynamics of Triton X-100 micelles induced by urea utilizing wavelength-selective fluorescence and related approaches using the fluorescent membrane probes NBD-PE (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoetha nolamine) and DPH (1,6-diphenyl-1,3,5-hexatriene). NBD-labeled lipids are widely used as fluorescent analogues of native lipids in biological and model membranes and membrane-mimetic systems to study a variety of processes.[42] In NBD-PE, the NBD group is covalently attached to the headgroup of a phosphatidylethanolamine molecule. The NBD group in NBD-PE is localized in the interfacial region of the membrane[43−48] and its location in the micellar environment is most likely to be interfacial. DPH and its derivatives represent popular membrane probes for monitoring organization and dynamics of the interior regions in membranes and micelles.[49] Our results utilizing several environment-sensitive fluorescence parameters show an increase in polarity in Triton X-100 micelles in the presence of increasing concentrations of urea. The mechanistic basis of the enhanced polarity is discussed.

Experimental Section

Materials. Triton X-100, ultrapure grade urea, cobalt chloride, MOPS (3-(*N*-morpholino)propanesulfonic acid) and DPH were purchased from Sigma Chemical Co. (St. Louis, MO). NBD-PE was obtained from Molecular Probes (Eugene, OR). Concentrations of stock solutions of NBD-PE and DPH in methanol were estimated using molar absorption coefficients (ϵ) of 21 000 M^{-1} cm^{-1} and 88 000 M^{-1} cm^{-1} at 463 and 350 nm, respectively.[33,50] The purity of NBD-PE was checked by thin-layer chromatography on silica gel precoated plates (Sigma) in chloroform/methanol/water (65:35:5, v/v/v) and was found pure when detected by its color or fluorescence. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout. The purity of Triton X-100 was checked by measuring its critical micelle concentration (CMC) value and comparing with literature CMC.

Determination of Critical Micelle Concentration of Triton X-100 Micelles. The CMC of Triton X-100 in the presence of varying concentrations of urea was determined fluorimetrically using a widely used method[51] previously developed by one of us. The method utilizes enhancement of DPH fluorescence upon micellization. Briefly, 1 μL of 10 mM DPH dissolved in tetrahydrofuran was added to various amounts of Triton X-100 dispersed in a total volume of 2 mL of aqueous solution containing appropriate amounts of urea. Tubes were incubated for 30 min in the dark at room temperature before measuring fluorescence. To reverse any photoisomerization of DPH, samples were kept in the dark in the fluorimeter for 30 s before the excitation shutter was opened and fluorescence measured. The excitation wavelength was 358 nm and emission was monitored at 430 nm. Excitation and emission slits with band-pass of 1.5 and 20 nm were used for all measurements. The excitation slit used was the minimum possible to minimize any photoisomerization of DPH during irradiation.

Sample Preparation. Concentrations of Triton X-100 used were double the CMC of Triton X-100 at any given concentration of urea to ensure that the detergent is in micellar state. The molar ratio of fluorophore to Triton X-100 was carefully chosen to give optimum signal-to-noise ratio with minimal perturbation to the micellar organization and negligible interprobe interactions. The ratio of fluorophore to Triton X-100 used was 1:1000 (mol/mol) in all cases. At such a low fluorophore-to-detergent molar ratio, not more than one probe molecule would be present per micelle on an average which rules out any probe aggregation effects, especially keeping in mind the aggregation number of ∼140 for Triton X-100.[52] Background samples were prepared the same way except that the fluorophore was not added to them. All experiments were done at room temperature (23 °C). Samples were equilibrated in the dark for an hour before measuring fluorescence.

Steady-State Fluorescence Measurements. Steady-state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes. Excitation and emission slits with a nominal band-pass of 5 nm were used for all measurements except in experiments involving DPH where slits with band-pass of 1.5 and 20 nm were used. Background intensities of samples in which NBD-PE was omitted were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts. The spectral shifts obtained with different sets of samples were identical in most cases. In other cases, the values were within ±1 nm of the ones reported. Fluorescence anisotropy measurements were performed using a Hitachi polarization accessory. Anisotropy values were calculated from the equation:[53]

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and emission polarizer vertically and horizontally oriented, respectively. G is the instrumental correction factor and is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light and is equal to I_{HV}/I_{HH}. All experiments were done at 23 °C with multiple sets of samples and average values of anisotropy shown in Tables 1 and 2 and Figure 3.

Time-Resolved Fluorescence Measurements. Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using a Photon Technology International (London, Western Ontario, Canada) LS-100 luminescence spectrophotometer in the time-correlated single photon counting mode. This machine uses a thyratron-gated nanosecond flash lamp filled with nitrogen as the plasma gas (16 ± 1 in. of mercury vacuum) and is run at 22−25 kHz. Lamp profiles were measured at the excitation wavelength using Ludox (colloidal silica) as the scatterer. To optimize the signal-to-noise ratio, 10 000 photon counts were collected in the peak channel. All experiments were performed using excitation and emission slits

with a nominal band-pass of 4 nm or less (excitation slit used was 1.5 nm for experiments with DPH). The sample and the scatterer were alternated after every 5% acquisition to ensure compensation for shape and timing drifts occurring during the period of data collection. This arrangement also prevents any prolonged exposure of the sample to the excitation beam thereby avoiding any possible photodamage of the fluorophore. Nevertheless, we recorded the fluorescence emission spectra of the fluorophore before and after the time-resolved data acquisition, and the data remained unchanged indicating no photodamage during data acquisition. This is an important control since both NBD-labeled lipids and DPH have previously been reported to be photobleached under certain conditions.[42,51] The data stored in a multichannel analyzer was routinely transferred to an IBM PC for analysis. Intensity decay curves so obtained were fitted as a sum of exponential terms:

$$F(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (2)$$

where α_i is a preexponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_i. The decay parameters were recovered using a nonlinear least squares iterative fitting procedure based on the Marquardt algorithm.[54] The program also includes statistical and plotting subroutine packages.[55] The goodness of the fit of a given set of observed data and the chosen function was evaluated by the reduced χ^2 ratio, the weighted residuals,[56] and the autocorrelation function of the weighted residuals.[57] A fit was considered acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation about zero with a minimum χ^2 value not more than 1.5. Mean (average) lifetimes $<\tau>$ for biexponential decays of fluorescence were calculated from the decay times and preexponential factors using the following equation:[53]

$$\langle\tau\rangle = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2} \quad (3)$$

Fluorescence Quenching Measurements. For quenching experiments using Co^{2+}, the fluorescence intensities of NBD-PE in Triton X-100 micelles containing varying concentrations of urea were measured in separate samples containing increasing concentrations of Co^{2+} taken from a freshly prepared stock solution in 10 mM MOPS, 150 mM NaCl, pH 7.2 buffer. Depending on the urea concentration, the quencher concentration was varied so that quenching of NBD fluorescence by Co^{2+} showed linear dependence in each case. Samples were incubated in dark for 12 h before measuring fluorescence. Corrections for inner filter effect were made using the following equation:[53]

$$F = F_{obs}\ \text{antilog}\ [(A_{ex} + A_{em})/2] \quad (4)$$

where F is the corrected fluorescence intensity and F_{obs} is the background subtracted fluorescence intensity of the sample. A_{ex} and A_{em} are the measured absorbance at the excitation and emission wavelengths. The absorbance of the samples was measured using a Hitachi U-2000 UV−visible absorption spectrophotometer. Quenching data were analyzed by fitting to the Stern−Volmer equation:[53]

$$F_o/F = 1 + K_{SV}[Q] = 1 + k_q\tau_o[Q] \quad (5)$$

where F_o and F are the fluorescence intensities in the absence and presence of the quencher, respectively, [Q] is the molar quencher concentration, and K_{SV} is the Stern−Volmer quenching constant. The Stern−Volmer quenching constant K_{SV} is equal to $k_q\tau_o$ where k_q is the bimolecular quenching constant and τ_o is the lifetime of the fluorophore in the absence of quencher.

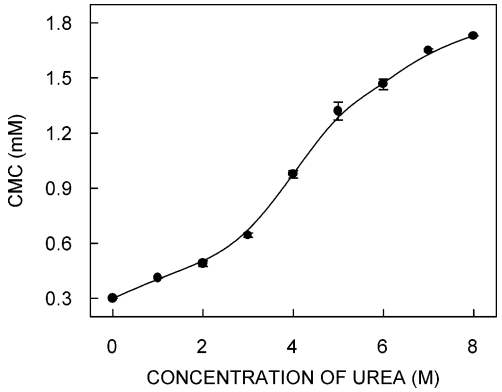

Figure 1. CMC of Triton X-100 micelles as a function of urea concentration determined fluorimetrically utilizing enhancement of DPH fluorescence upon micellization. See Experimental Section for other details.

Results

Effect of Urea on the CMC of Triton X-100. The principle of the DPH assay of CMC is that DPH fluorescence will be greatly enhanced above the CMC because of its incorporation into the hydrophobic interior of the micelle. A unique advantage of this method is that, unlike some other methods, the CMC determined using this approach is reliable even for charged detergents. This is in contrast to methods which employ charged fluorescent probes where it has been demonstrated that such assays usually do not work if probe and detergent have opposite charges.[58]

Figure 1 shows the CMC of Triton X-100 determined using the DPH method as a function of urea concentration. The CMC of Triton X-100 in the absence of urea is 0.3 mM which is in excellent agreement with the literature value.[51,52] However, the CMC increases in the presence of varying concentrations of urea. Thus, the CMC shows ~6-fold increase from 0.3 to 1.72 mM upon increasing urea concentration up to 8 M (Figure 1). We attribute the increase in CMC with increasing urea concentration to the ability of urea to weaken hydrophobic force responsible for the formation and maintenance of the micellar assembly in aqueous solutions. For all subsequent experiments, concentrations of Triton X-100 used were double the CMC of Triton X-100 at the respective urea concentration. This ensures that the detergent is in micellar state in all cases.

Fluorescence Emission Maximum of NBD-PE in Triton X-100 Micelles. The fluorescence emission maximum of NBD-PE in Triton X-100 micelles as a function of increasing urea concentration is shown in Table 1. The maximum of fluorescence emission of NBD-PE in Triton X-100 micelles in absence of urea is 529 nm which is in agreement with our previously reported value.[17] This is similar to the emission maximum observed in membranes (530 nm)[30,44] where the NBD-PE is localized in the motionally restricted interfacial region.[43−48] Table 1 shows that the fluorescence emission maximum of NBD-PE undergoes a progressive red shift of 4 nm (from 529 to 533 nm) when the concentration of urea is increased from 0 to 8 M. This suggests that there is an increase in polarity around the NBD group of NBD-PE when bound to Triton X-100 micelles in the presence of urea.

Red Edge Excitation Shift of Micelle-Bound NBD-PE. The shifts in the maxima of fluorescence emission[59] of NBD-PE

TABLE 1: Emission Maximum and Anisotropy of NBD-PE in Triton X-100 Micelles as a Function of Urea Concentration[a]

[urea] (M)	emission maximum (nm)	fluorescence anisotropy[b]
0	529	0.130 ± 0.001
2	530	0.137 ± 0.001
4	531	0.139 ± 0.001
6	532	0.151 ± 0.002
8	533	0.159 ± 0.001

[a] The excitation wavelength used was 465 nm. The ratio of NBD-PE to Triton X-100 was 1:1000 (mol/mol) and the concentration of NBD-PE ranged from 0.6 to 3.5 μM depending on the concentration of urea used. See Experimental Section for other details. [b] Calculated using eq 1. Anisotropy values were recorded at 531 nm and the excitation wavelength used was 465 nm. The data shown represent the means ± standard error of three independent measurements.

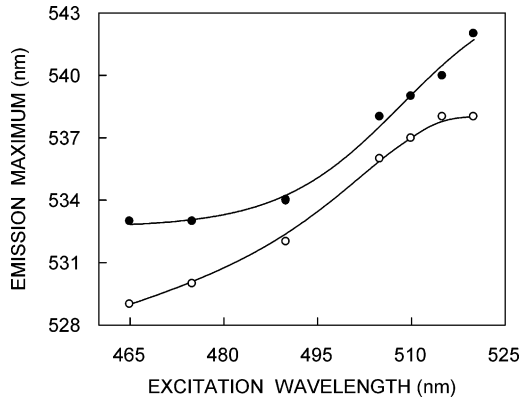

Figure 2. Effect of changing excitation wavelength on the wavelength of maximum emission for NBD-PE in Triton X-100 micelles in the absence (○) and presence of 8 M (●) urea. All other conditions are as in Table 1. See Experimental Section for other details.

when bound to Triton X-100 micelles in the absence and presence of urea as a function of excitation wavelength are shown in Figure 2. As can be seen from the figure, upon excitation at 465 nm, the fluorescence emission maxima of NBD-PE is at 529 and 533 nm in Triton X-100 micelles in the absence and presence of 8 M urea, respectively. The value of these emission maxima suggests that the NBD group in NBD-PE is localized at the interfacial region of the micelles. As the excitation wavelength is changed from 465 to 520 nm, the emission maxima of micelle-bound NBD-PE are shifted from 529 to 538 nm (in the absence of urea), and 533 to 542 nm (in the presence of 8 M urea), which correspond to REES of 9 nm in each case. The magnitude of REES was 9 nm even when intermediate concentrations of urea were used between 0 and 8 M (not shown). Such dependence of the emission spectra on the excitation wavelength is characteristic of red edge effect. This in turn implies that the NBD moiety in NBD-PE, when incorporated in Triton X-100 micelles, is in an environment where its mobility is considerably reduced. Since the NBD group is localized in the interfacial region (see above), such a result would directly imply that this region of the micelle offers considerable restriction to the reorientational motion of the solvent dipoles around the excited-state fluorophore. Interestingly, the magnitude of REES is independent of the presence of urea. This shows that the presence of urea does not change the restriction to solvent reorientation in the excited state although the emission maximum shows a red shift of 4 nm because of increased polarity in the presence of urea.

Fluorescence Anisotropy of Micelle-Bound Probes. The fluorescence anisotropy of NBD-PE in Triton X-100 micelles

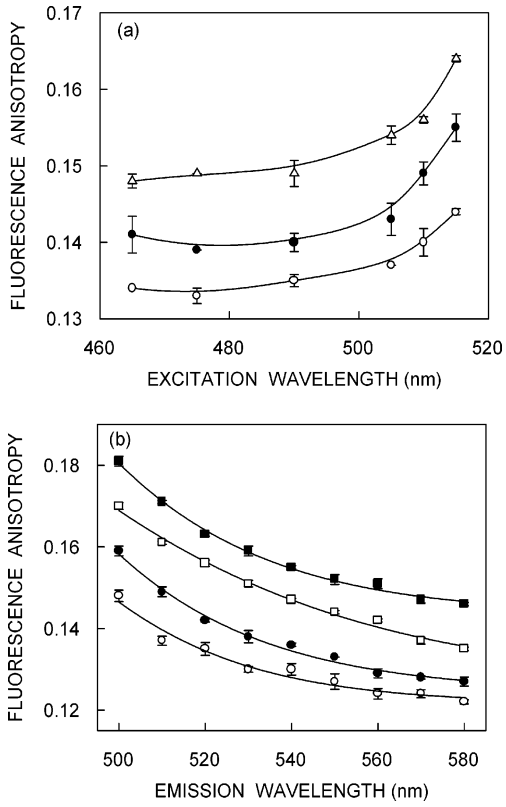

Figure 3. (a) Fluorescence anisotropy of NBD-PE in Triton X-100 micelles in the absence (○) and presence of 2 M (●) and 6 M (△) urea as a function of excitation wavelength. Anisotropy values were recorded at 531 nm. (b) Fluorescence anisotropy of NBD-PE in Triton X-100 micelles in the absence (○) and presence of 4 M (●), 6 M (□), and 8 M (■) urea as a function of emission wavelength. The excitation wavelength used was 465 nm. The data points shown are the means of three independent measurements. The error bars represent the standard error. All other conditions are as in Table 1. See Experimental Section for other details.

as a function of urea concentration is shown in Table 1. The fluorescence anisotropy of NBD-PE shows a steady increase in the presence of increasing concentrations of urea. This suggests that the rotational mobility of NBD-PE is considerably reduced in the presence of urea. This supports our earlier conclusion that the NBD-PE is in a motionally restricted region when bound to micelles. The increase in the rotational restriction with increasing urea concentration could be due to increased hydrogen bonding of the NBD group to urea because of its presence in the immediate vicinity. In fact, it has recently been shown that urea can induce enhanced solvation of polar groups in an amphiphile.[60] However, there are other factors that need to be considered while interpreting changes in fluorescence anisotropy with urea concentration. An important factor is the viscosity of the medium which increases considerably at higher urea concentrations. This could also contribute to increase in anisotropy with increasing urea concentration. However, an increase in medium viscosity generally brings about an increase in fluorescence lifetime.[61,62] Interestingly, we observe a decrease in mean fluorescence lifetime in this case (see later, Table 3). We therefore rule out viscosity change as a possible cause for the observed changes in anisotropy with urea concentration.

Fluorescence anisotropy is also known to be dependent on excitation wavelength in motionally restricted media.[19] A plot of steady-state anisotropy of NBD-PE incorporated in Triton X-100 micelles as a function of excitation wavelength is shown in Figure 3a for various concentrations of urea. The observed increase in anisotropy value strengthens our earlier conclusion

TABLE 2: Anisotropy and Rotational Correlation Times of DPH in Triton X-100 Micelles as a Function of Urea Concentration

[urea] (M)	fluorescence anisotropy[a]	τ_c[b] (ns)
0	0.147 ± 0.002	5.52
1	0.149 ± 0.001	5.54
2	0.157 ± 0.002	6.00
3	0.157 ± 0.001	5.93
4	0.163 ± 0.002	6.17
5	0.166 ± 0.003	6.28
6	0.179 ± 0.001	7.11
7	0.180 ± 0.001	7.16
8	0.187 ± 0.001	7.74

[a] Calculated using eq 1. Anisotropy values were recorded at 430 nm and the excitation wavelength used was 358 nm. The data shown represent the means ± standard error of three independent measurements. All other conditions are in Table 1. See Experimental Section for other details. [b] Calculated using eq 6.

that the NBD group of NBD-PE is localized in a motionally restricted environment in this micellar system and this increase in anisotropy is more pronounced at higher urea concentrations. This clearly shows, in agreement with Table 1, the increased motional restriction of NBD-PE as the concentration of urea is increased.

In addition, we monitored the change in fluorescence anisotropy of NBD-PE in Triton X-100 micelles as a function of emission wavelength (see Figure 3b). There is a considerable reduction in fluorescence anisotropy upon increasing the emission wavelength irrespective of the presence of urea in Triton X-100 micelles. The lowest anisotropy is observed toward longer wavelengths (red edge) where emission from the relaxed fluorophores predominates. Taken together, these results support that the NBD moiety of NBD-PE in Triton X-100 micelles experiences increased motional restriction upon increasing the urea content.

Although the exact shape of Triton X-100 micelles is not yet resolved, the most likely shape is that of an oblate sphere which would display considerable anisotropy in its organization.[63] A comprehensive description of any change in the organization and dynamics of such an organized assembly should include information from probes which are localized in the deeper hydrophobic core region of the micelle. We therefore used a fluorescent probe DPH, which would partition to deeper regions of the micelle, to characterize the changes taking place in these regions in the presence of increasing concentrations of urea.

Table 2 shows the fluorescence anisotropy of DPH incorporated in Triton X-100 micelles with increasing urea concentrations. The plot shows a steady increase in the presence of increasing concentrations of urea suggesting that the rotational mobility of DPH is considerably reduced in the presence of urea. The size of nonionic micelles such as Triton X-100 have previously been shown to be reduced in the presence of urea.[64] The decrease in rotational mobility of DPH in Triton X-100 micelles in the presence of urea could be due to the packing constraints generated in the interior of the micelles because of reduction in size.

Time-Resolved Fluorescence Measurements of Micelle-Bound Probes. Fluorescence lifetime serves as a sensitive indicator of the local environment in which a given fluorophore is placed.[65] In addition, it is well known that fluorescence lifetime of the NBD group is sensitive to the environment in which it is placed.[66] Any change in the microenvironment of a micelle-bound probe therefore could be expected to give rise to differences in fluorescence lifetime. The fluorescence lifetimes of micelle-bound NBD-PE at various concentrations of urea are shown in Table 3. As seen from the table, all fluorescence decays could be fitted well with a biexponential function. The mean fluorescence lifetimes of NBD-PE in Triton X-100 micelles were calculated using eq 3 and are plotted as a function of increasing urea concentration in Table 3. As shown in the table, there is a continuous decrease in mean lifetime of NBD-PE with increasing urea concentration in the Triton X-100 micelles. Thus, the mean lifetime decreases from ∼5.3 to ∼4.4 ns (i.e., by ∼17%) when urea concentration is increased from 0 to 8 M.

TABLE 3: Lifetimes of NBD-PE in Triton X-100 Micelles as a Function of Urea Concentration[a]

[urea] (M)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle$[b] (ns)	χ^2
0	0.15	8.27	0.85	4.36	5.34	1.28
1	0.11	8.21	0.89	4.44	5.14	1.27
2	0.11	8.49	0.89	4.14	5.02	1.26
3	0.21	7.06	0.79	3.82	4.89	1.29
4	0.12	7.97	0.88	3.83	4.75	1.33
5	0.26	6.36	0.74	3.55	4.64	1.27
6	0.24	6.59	0.76	3.35	4.59	1.26
7	0.28	6.28	0.72	3.31	4.57	1.28
8	0.22	6.67	0.78	3.16	4.47	1.30

[a] The excitation wavelength was 465 nm; emission was monitored at 531 nm. All other conditions are as in Table 1. [b] Calculated using eq 3.

TABLE 4: Lifetimes of DPH in Triton X-100 Micelles as a Function of Urea Concentration[a]

[urea] (M)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle$[b] (ns)	χ^2
0	0.33	9.48	0.67	7.01	8.00	1.21
1	0.39	9.11	0.61	6.77	7.85	1.23
2	0.35	9.16	0.65	6.73	7.76	1.09
3	0.30	9.35	0.70	6.65	7.67	1.23
4	0.44	8.75	0.56	5.97	7.46	1.13
5	0.43	8.60	0.57	5.98	7.34	1.25
6	0.63	8.01	0.37	4.94	7.19	1.26
7	0.43	8.53	0.57	5.58	7.16	1.14
8	0.36	8.74	0.64	5.82	7.16	1.26

[a] The excitation wavelength was 358 nm; emission was monitored at 430 nm. All other conditions are as in Table 1. [b] Calculated using eq 3.

The lifetime of NBD-PE incorporated in various membranes have been reported to be ∼7 ns.[30,67] In water, however, NBD lifetime reduces to ∼1.5 ns, which has been attributed to an increase in polarity as well as the nature of hydrogen-bonding interactions between the fluorophore and the solvent[66] which is accompanied by an increase in the rate of nonradiative decay.[68] Thus, the observed reduction in fluorescence lifetime of NBD-PE with increasing urea concentration could possibly be attributed to an increase in polarity of the microenvironment and any possible alteration in hydrogen-bonding interactions around the NBD group of NBD-PE (supported by data from Table 1).

DPH fluorescence is environmentally sensitive[69] and has earlier been used to probe the water content of membranes since its lifetime is reduced with increasing water content in its immediate environment.[70] To explore changes in the interior of the micelle, we measured the change in fluorescence lifetime of the deeper probe DPH as a function of urea concentration (see Table 4). All fluorescence decays for micelle-bound DPH could be fitted well with a biexponential function. Table 4 shows that there is a continuous decrease in mean fluorescence lifetime of DPH with increasing urea concentration. Thus, the mean lifetime decreases from ∼8 to ∼7.1 ns (i.e., by ∼11%) when

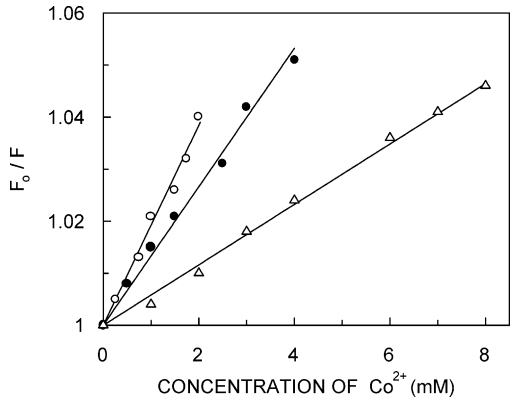

Figure 4. Representative data for Stern−Volmer analysis of Co^{2+} quenching of NBD-PE fluorescence in Triton X-100 micelles in the absence (△) and presence of 6 M (●) and 8 M (○) urea. F_o is the fluorescence in the absence of quencher, F is the corrected fluorescence in the presence of quencher. The excitation wavelength was 465 nm and emission was monitored at 531 nm. All other conditions are as in Table 1. See Experimental Section for other details.

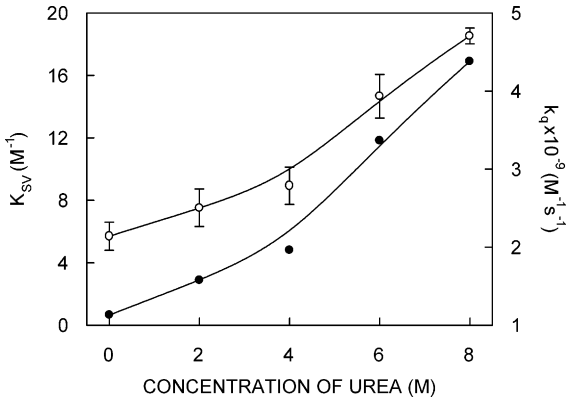

Figure 5. Stern−Volmer quenching constants, K_{SV} (○) and bimolecular quenching constants, k_q (●) for Co^{2+} quenching of NBD-PE incorporated in Triton X-100 micelles as a function of urea concentration. The excitation wavelength was 465 nm and emission was monitoored at 531 nm. All other conditions are as in Table 1. See Experimental Section for other details.

urea concentration is increased from 0 to 8 M. This could be due to increased polarity in the micelle interior induced by urea.

To ensure that the observed change in steady-state anisotropy of DPH as a function of urea concentration is not due to any change in lifetime with increasing urea concentration (see above and Table 2), the apparent (average) rotational correlation times for DPH in Triton X-100 micelles with increasing concentrations of urea were calculated using Perrin's equation:[53]

$$\tau_c = \frac{<\tau>r}{r_o - r} \tag{6}$$

where r_o is the limiting anisotropy of DPH, r is the steady-state anisotropy, and $<\tau>$ is the mean fluorescence lifetime as calculated from eq 3. Although Perrin's equation is not strictly applicable to this system, it is assumed that this equation will apply to a first approximation, especially because we have used mean fluorescence lifetimes for the analysis of multiple component lifetimes. The values of the apparent rotational correlation times, calculated this way using a value of r_o of 0.36,[71] are shown in Table 2. There is a considerable increase in rotational correlation times from ∼5.5 to 7.7 ns (i.e., by ∼39%) of DPH with increasing concentrations of urea which clearly shows that the observed change in anisotropy values (Table 2) were not due to any lifetime-induced artifacts.

Accessibility Studies of Micelle-Bound NBD-PE. The results presented above show that increasing concentrations of urea enhance the polarity of the micelles and induce increased hydration in the micelle. To examine the accessibility and relative location of NBD-PE and confirm that the presence of urea increases water penetration in Triton X-100 micelles, fluorescence quenching experiments were performed with the aqueous quencher Co^{2+}. The paramagnetic cobaltous ion is soluble in water and is an efficient quencher of NBD fluorescence.[18,44,72,73] The results of these experiments for quenching of NBD-PE by Co^{2+} in Triton X-100 micelles containing different amounts of urea are plotted in Figure 4 as Stern−Volmer plots. The slope of such a plot (K_{SV}) is related to the degree of exposure (accessibility) of the NBD group to the aqueous phase. In general, the higher the slope, the greater the degree of exposure, assuming that there is not a large difference in fluorescence lifetime. The bimolecular quenching constant (k_q) is a more accurate measure of the degree of exposure since it takes into account the differences in fluorescence lifetime (see eq 5). The quenching parameters obtained by analyzing the Stern−Volmer plots (both K_{SV} and k_q) are plotted in Figure 5. It is encouraging to note from the figure that the bimolecular quenching constants (k_q) are in overall agreement with the Stern−Volmer constants (K_{SV}) implying that the conclusions derived from the analysis of Stern−Volmer constants are not influenced by changes in lifetime. It is apparent from the figure that increasing urea concentration increases the accessibility of NBD group of NBD-PE to Co^{2+}, irrespective of the parameter chosen (K_{SV} or k_q). Maximum quenching of NBD-PE is observed in Triton X-100 micelles containing 8 M urea clearly indicating that presence of urea increases micellar water penetration which in turn results in an increased polarity.

Discussion

The stability of organized molecular assemblies such as micelles is determined by the delicate balance between hydrophilic and hydrophobic forces. In aqueous solution, the balance between hydrophilic and hydrophobic forces and hence the stability of micelles can be modulated by modifying the properties of the aqueous medium. A convenient way to achieve this is by using chemical agents that affect the properties of the aqueous solvent by interacting with it. Chaotropes such as urea perturb the structure of organized molecular assemblies such as micelles.[10,11] Traditionally, this has been thought to be due to the ability of urea to act as a "water structure breaker".[74] However, recent evidence suggests that the ability of urea to perturb nonionic micellar structures is dependent on either replacement of water molecules in the hydration shell of the solute[12] or by enhancement of the hydrophilic properties of water which results in more strongly solvated polar headgroups of the constituent monomers of the micelle.[60] The latter mechanism suggests that urea−water mixture solvates the polar groups in micellar aggregates better than water alone.

In this report, we have monitored the change in the organization and dynamics of the nonionic Triton X-100 micelles induced by urea utilizing wavelength-selective fluorescence and related approaches using the fluorescent membrane probes NBD-PE and DPH. The NBD group represents a widely used extrinsic fluorophore in biophysical, biochemical, and cell biological studies (for a review, see ref 42). NBD-labeled lipids are extensively used as fluorescent analogues of native lipids in biological and model membranes to study a variety of processes.[33,44,68,75] The NBD moiety possesses some of the most

desirable properties for serving as an excellent probe for both spectroscopic and microscopic applications. It is very weakly fluorescent in water. Upon transfer to a hydrophobic medium, it fluoresces brightly in the visible range and exhibits a high degree of environmental sensitivity.[30,44,66,76,77] Fluorescence lifetime of the NBD group is extremely sensitive to the environmental polarity.[18,38,66] It is relatively photostable, and lipids labeled with the NBD group mimic endogenous lipids in studies of intracellular lipid transport.[42] More importantly, we have earlier shown using solvatochromic and quantum chemical approaches that the dipole moment of the NBD group changes by ~4 D upon excitation,[76] an important criterion for a fluorophore to exhibit REES effects.[19,22] DPH and its derivatives, on the other hand, represent popular membrane probes for monitoring organization and dynamics of the interior regions in membranes and micelles.[49] The large degree of environmental sensitivity of NBD and DPH fluorescence is useful in monitoring micellar organization under various conditions. For example, the observation that NBD[38,66] and DPH[69,70] lifetimes are shortened in polar environment offers a convenient way to study micellar organization with varying concentrations of urea.

Our results utilizing several sensitive fluorescence parameters show increased polarity in Triton X-100 micelles in the presence of varying concentrations of urea. This could possibly be due to increased water penetration in Triton X-100 micelles induced by urea. This is supported by fluorescence quenching experiments which indicate increased accessibility from the aqueous phase at higher urea concentrations. In addition, this could result from better solvation of the polar headgroups by urea−water mixture than water alone.

Acknowledgment. This work was supported by the Council of Scientific and Industrial Research, Government of India. We thank Y.S.S.V. Prasad and G.G. Kingi for technical help and members of our laboratory for critically reading the manuscript. H.R. thanks the Council of Scientific and Industrial Research for the award of a Senior Research Fellowship. S.K.P. was awarded a Summer Training Program Fellowship by the Centre for Cellular and Molecular Biology.

References and Notes

(1) Helenius, A.; Simons, K. *Biochim. Biophys. Acta* **1975**, *415*, 29.
(2) Banerjee, P. In *Encyclopedia of Molecular Biology*; Creighton, T. E., Ed.; John Wiley: New York, 1999; pp 661−666.
(3) Chattopadhyay, A.; Harikumar, K. G.; Kalipatnapu, S. *Mol. Membr. Biol.* **2002**, *19*, 211.
(4) Tanford, C. *Science* **1978**, *200*, 1012.
(5) Israelachvili, J. N.; Marcelja, S.; Horn, R. G. *Q. Rev. Biophys.* **1980**, *13*, 121.
(6) Tanford, C. *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*; Wiley-Interscience: New York, 1980.
(7) Tanford, C. *Biochem. Soc. Trans.* **1987**, *15*, 1S.
(8) Menger, F. M. *Acc. Chem. Res.* **1979**, *12*, 111.
(9) Lindman, B.; Wennerstrom, H. In *Solution Behavior of Surfactants: Theoretical and Applied Aspects*; Mittal, K. L., Fendler, E. J., Eds.; Plenum Press: New York, 1982; Vol. 1, pp 3−25.
(10) Makhatadze, G. I.; Privalov, P. L. *J. Mol. Biol.* **1992**, *226*, 491.
(11) Midura, R. J.; Yanagishita, M. *Anal. Biochem.* **1995**, *228*, 318.
(12) Ruiz, C. C.; Sánchez, F. G. *J. Colloid Interface Sci.* **1994**, *165*, 110.
(13) Costantino, L.; D'Errico, G.; Roscigno, P.; Vitagliano, V. *J. Phys. Chem. B* **2000**, *104*, 7326.
(14) Brown, D. A.; Rose, J. K. *Cell* **1992**, *68*, 533.
(15) Simons, J. W. F. A.; Boots, J. W. P.; Kats, M. P.; Slotboom, A. J.; Egmond, M. R.; Verheij, H. M. *Biochemistry* **1997**, *36*, 14539.
(16) Acharya, P.; Rao, N. M. *Langmuir* **2002**, *18*, 3018.
(17) Rawat, S. S.; Mukherjee, S.; Chattopadhyay, A. *J. Phys. Chem. B* **1997**, *101*, 1922.
(18) Rawat, S. S.; Chattopadhyay, A. *J. Fluoresc.* **1999**, *9*, 233.
(19) Mukherjee, S.; Chattopadhyay, A. *J. Fluoresc.* **1995**, *5*, 237.
(20) Chattopadhyay, A. In *Fluorescence Spectroscopy, Imaging and Probes*; Kraayenhof, R., Visser, A. J. W. G., Gerritsen, H. C., Eds.; Springer-Verlag: Heidelberg, Germany, 2002; pp 211−224.
(21) Raghuraman, H.; Kelkar, D. A.; Chattopadhyay, A. *Proc. Ind. Natl. Sci. Acad. A* **2003**, *69*, 25.
(22) Chattopadhyay, A. *Chem. Phys. Lipids* **2003**, *122*, 3.
(23) Demchenko, A. P. *Trends Biochem. Sci.* **1988**, *13*, 374.
(24) Demchenko, A. P. *Luminescence* **2002**, *17*, 19.
(25) Galley, W. C.; Purkey, R. M. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *67*, 1116.
(26) Lakowicz, J. R.; Keating-Nakamoto, S. *Biochemistry* **1984**, *23*, 3013.
(27) Israelachvili, J.; Wennerstörm, H. *Nature* **1996**, *379*, 219.
(28) Ghosh, A. K.; Rukmini, R.; Chattopadhyay, A. *Biochemistry* **1997**, *36*, 14291.
(29) Chattopadhyay, A.; Rukmini, R. *FEBS Lett.* **1993**, *335*, 341.
(30) Chattopadhyay, A.; Mukherjee, S. *Biochemistry* **1993**, *32*, 3804.
(31) Mukherjee, S.; Chattopadhyay, A. *Biochemistry* **1994**, *33*, 5089.
(32) Chattopadhyay, A.; Mukherjee, S.; Rukmini, R.; Rawat, S. S.; Sudha, S. *Biophys. J.* **1997**, *73*, 839.
(33) Chattopadhyay, A.; Mukherjee, S. *J. Phys. Chem. B* **1999**, *103*, 8180.
(34) Chattopadhyay, A.; Mukherjee, S. *Langmuir* **1999**, *15*, 2142.
(35) Raja, S. M.; Rawat, S. S.; Chattopadhyay, A.; Lala, A. K. *Biophys. J.* **1999**, *76*, 1469.
(36) Kelkar, D. A.; Ghosh, A.; Chattopadhyay, A. *J. Fluoresc.* **2003**, *13*, 459.
(37) Mukherjee, S.; Raghuraman, H.; Dasgupta, S.; Chattopadhyay, A. *Chem. Phys. Lipids* **2004**, *127*, 91.
(38) Chattopadhyay, A.; Mukherjee, S.; Raghuraman, H. *J. Phys. Chem. B* **2002**, *106*, 13002.
(39) Raghuraman, H.; Chattopadhyay, A. *Langmuir* **2003**, *19*, 10332.
(40) Guha, S.; Rawat, S. S.; Chattopadhyay, A.; Bhattacharyya, B. *Biochemistry* **1996**, *35*, 13426.
(41) Chattopadhyay, A.; Rawat, S. S.; Kelkar, D. A.; Ray, S.; Chakrabarti, A. *Protein Sci.* **2003**, *12*, 2389.
(42) Chattopadhyay, A. *Chem. Phys. Lipids* **1990**, *53*, 1.
(43) Chattopadhyay, A.; London, E. *Biochemistry* **1987**, *26*, 39.
(44) Chattopadhyay, A.; London, E. *Biochim. Biophys. Acta* **1988**, *938*, 24.
(45) Pagano, R. E.; Martin, O. C. *Biochemistry* **1988**, *27*, 4439.
(46) Mitra, B.; Hammes, G. G. *Biochemistry* **1990**, *29*, 9879.
(47) Wolf, D. E.; Winiski, A. P.; Ting, A. E.; Bocian, K. M.; Pagano, R. E. *Biochemistry* **1992**, *31*, 2865.
(48) Abrams, F. S.; London, E. *Biochemistry* **1993**, *32*, 10826.
(49) Lentz, B. R. *Chem. Phys. Lipids* **1989**, *50*, 171.
(50) *Handbook of Fluorescent Probes and Research Chemicals*, 6th ed.; Haugland, R. P., Ed.; Molecular Probes: Eugene, OR, 1996.
(51) Chattopadhyay, A.; London, E. *Anal. Biochem.* **1984**, *139*, 408.
(52) Neugebauer, J. M. *Methods Enzymol.* **1990**, *182*, 239.
(53) Lakowicz, J. R. In *Principles of Fluorescence Spectroscopy*; Kluwer-Plenum Press: New York, 1999.
(54) Bevington, P. R. In *Data Reduction and Error Analysis for the Physical Sciences*; McGraw-Hill: New York, 1969.
(55) O'Connor, D. V.; Phillips, D. In *Time-Correlated Single Photon Counting*; Academic Press: London, 1984; pp 180−189.
(56) Lampert, R. A.; Chewter, L. A.; Phillips, D.; O'Connor, D. V.; Roberts, A. J.; Meech, S. R. *Anal. Chem.* **1983**, *55*, 68.
(57) Grinvald, A.; Steinberg, I. Z. *Anal. Biochem.* **1974**, *59*, 583.
(58) Vendittis, E. D.; Palumbo, G.; Parlato, G.; Bocchini, V. *Anal. Biochem.* **1981**, *115*, 278.
(59) We have used the term maximum of fluorescence emission in a somewhat wider sense here. In every case, we have monitored the wavelength corresponding to maximum fluorescence intensity, as well as the center of mass (i.e., the weighted average of fluorescence intensity as a function of emission wavelength) of the fluorescence emission. In most cases, both these methods yielded the same wavelength. In cases where minor discrepancies were found, the center of mass of emission has been reported as the fluorescence maximum.
(60) Dias, G. L.; Florenzano, F. H.; Reed, W. F.; Baptista, M. S.; Souza, S. M. B.; Alvarez, E. B.; Chaimovich, H.; Cuccovia, I. M.; Amaral, C. L. C.; Brasil, C. R.; Romsted, L. S.; Politi, M. J. *Langmuir* **2002**, *18*, 319.
(61) Tredwell, C. J.; Keary, C. M. *Chem. Phys.* **1979**, *43*, 307.
(62) Krishna, M. M. G.; Periasamy, N. *J. Fluoresc.* **1998**, *8*, 81.
(63) Paradies, H. H. *J. Phys. Chem.* **1980**, *84*, 599.
(64) Briganti, G.; Puvvada, S.; Blankschtein, D. *J. Phys. Chem.* **1991**, *95*, 8989.
(65) Prendergast, F. G. *Curr. Opin. Struct. Biol.* **1991**, *1*, 1054.
(66) Lin, S.; Struve, W. S. *Photochem. Photobiol.* **1991**, *54*, 361.
(67) Arvinte, T.; Cudd, A.; Hildenbrand, K. *Biochim. Biophys. Acta* **1986**, *860*, 215.
(68) Mazeres, S.; Schram, V.; Tocanne, J.-F.; Lopez, A. *Biophys. J.* **1996**, *71*, 327.

(69) Ho, C.; Stubbs, C. D. *Biophys. J.* **1992**, *63*, 897.
(70) Stubbs, C. D.; Ho, C.; Slater, S. J. *J. Fluoresc.* **1995**, *5*, 19.
(71) Shinitzky, M.; Barenholz, Y. *J. Biol. Chem*. **1974**, *249*, 2652.
(72) Homan, R.; Eisenberg, M. *Biochim. Biophys. Acta* **1985**, *812*, 485.
(73) Morris, S. J.; Bradley, D.; Blumenthal, R. *Biochim. Biophys. Acta* **1985**, *818*, 365.
(74) Finer, E. G.; Francks, F.; Tait, M. J. *J. Am. Chem. Soc*. **1974**, *94*, 4424.
(75) Liao, S.; Lin, J.; Do, H.; Johnson, A. E. *Cell* **1997**, *90*, 31.
(76) Mukherjee, S.; Chattopadhyay, A.; Samanta, A.; Soujanya, T. *J. Phys. Chem*. **1994**, *98*, 2809.
(77) Fery-Forgues, S.; Fayet, J.-P.; Lopez, A. *J. Photochem. Photobiol. A* **1993**, *70*, 229.