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Abstract
The coexistence of different excited states with different properties of the same chromophores could have significant consequences for the accurate characterization of solvation dynamics in a heterogeneous environment, such as a protein. The purpose of this work is to study the contributions of the locally excited (LE) and charge-transferred (CT) states of the fluorescent probe molecule 6-propionyl-2-(N,N-dimethylamino)naphthalene (PRODAN) to its solvation dynamics in the heterogeneous environment provided by reverse micelles formed by sodium 1,4-bis-(2-ethylhexyl) sulfosuccinate (AOT)/n-heptane/water. We have found that the LE and CT states of PRODAN solvate on different time scales in reverse micelles (2 and ~0.4 ns, respectively), consistent with results suggested in the literature, and have concluded that PRODAN’s use as a probe of heterogeneous environments must be used with caution and that, more importantly, the same caution must be exercised with any chromophore capable of emitting from different excited states.

Keywords
excited states, fluorescence, micelles, naphthalene, polymer blends, probes, sodium, fluorescent probe molecules, heterogeneous environments, reverse micelles, solvation dynamics

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Solvation Dynamics of the Fluorescent Probe PRODAN in Heterogeneous Environments: Contributions from the Locally Excited and Charge-Transferred States

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The coexistence of different excited states with different properties of the same chromophores could have significant consequences for the accurate characterization of solvation dynamics in a heterogeneous environment, such as a protein. The purpose of this work is to study the contributions of the locally excited (LE) and charge-transferred (CT) states of the fluorescent probe molecule 6-propionyl-2-(N,N-dimethylamino)naphthalene (PRODAN) to its solvation dynamics in the heterogeneous environment provided by reverse micelles formed by sodium 1,4-bis-(2-ethylhexyl) sulfosuccinate (AOT)/n-heptane/water. We have found that the LE and CT states of PRODAN solvate on different time scales in reverse micelles (2 and ~0.4 ns, respectively), consistent with results suggested in the literature, and have concluded that PRODAN’s use as a probe of heterogeneous environments must be used with caution and that, more importantly, the same caution must be exercised with any chromophore capable of emitting from different excited states.

Introduction

Fluorescent molecules sensitive to environmental polarities have been used extensively as probes in the studies of physicochemical properties of solvents, surfaces, proteins, membranes, cells, etc.1–5 It is critical to understand the photophysical properties of the fluorescent probe molecule, especially if it can emit from more than one state. 6-Propionyl-2-(N,N-dimethylamino)naphthalene (PRODAN) (Figure 1) is a highly fluorescent, hydrophobic molecule, first synthesized and characterized by Weber and Farris in 1979.6 Its absorption and emission spectra are strongly dependent upon the polarity of its environment,6 and it has a long history of use in probing biological systems.7–14 It has been used as an optical probe of the function and dynamics of proteins and membranes.7–14 Recently, it has also been used to study solvation dynamics in polar liquids, ionic liquids, and supercritical fluids.15–19

PRODAN is a push–pull, charge-transfer chromophore that produces a substantial change in its excited-state dipole moment upon photoexcitation owing to the presence of an electron-donating dimethylamino group and electron-withdrawing propionyl group connected to the aromatic spacer by a single bond. The excited-state kinetics of PRODAN are complex, and the origins of its solvatochromatic nature have been debated.20–26 Theoretical calculations suggest a planar structure in the ground state. However, the geometry of its charge-transferred emissive state has not yet been confirmed.20,27 Several theoretical studies have been performed to determine the possible conformational changes in its excited-state geometry, for example, whether the emissive state is twisted intramolecular charge transfer (TICT) or planar intramolecular charge transfer (PICT).20–23,35

Lakowicz and Balter36,37 studied PRODAN in n-butanol and suggested that its spectral relaxation requires at least two steps. Later, Heisel et al.28 explained the time-resolved fluorescence experimental results of PRODAN in n-butanol in terms of a nonradiative intramolecular reaction from a locally excited state to an energetically lower charge-transferred state and to solute–solvent interactions. Chapman et al.35 and Chapman and

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Figure 1. Structure of (a) 6-propionyl-2-(N,N-dimethylamino)naphthalene (PRODAN), (b) 2′-(N,N-dimethylamino)-6-naphthyl-4-trans-cyclohexanoic acid (DANCA), and (c) Aladan.

Figure 2. The charge-transferred (CT) state is created via the locally excited (LE) state in polar environments.

Maroccelli16 discussed the spectral relaxation of PRODAN in terms of a continuous solvation process. It is generally agreed that PRODAN undergoes excited-state intramolecular charge transfer following excitation from the ground state. The charge-transferred state, CT, is formed from an initially excited state referred to as the locally excited state (LE).38 In nonpolar environments, emission is from the LE state, while in polar environments the emission is from the CT state (Figure 2).38

PRODAN has a single broad emission spectrum with a dramatic solvatochromatic shift (∼130 nm) of its maximum, ranging from 401 nm in cyclohexane to 531 nm in water.5 Weber and Farris6 first reported a change in dipole moment, ∆µ∼20 D, upon excitation, and concluded that this was responsible for its large Stokes shift. Balter et al. modified this value to 10 D and suggested that solvent-specific interactions (e.g., hydrogen bonding) may cause a large Stokes shift in polar protic solvents.25 Recently, Samanta et al.32 have suggested this
value to be 4.4–5.0 D based on transient dielectric loss measurements. Also, ground and excited Franck–Condon state dipole moments of PRODAN were recently reported from electrooptical absorption measurements.39

PRODAN has a single-exponential fluorescence lifetime decay in nonpolar solvents (e.g., cyclohexane, ∼0.2 ns; heptane, ∼0.14 ns) and in polar aprotic solvents (e.g., acetonitrile, ∼3.3 ns).22,40 However, it exhibits a biexponential lifetime in polar protic solvents such as methanol and water. The reported lifetime components in methanol are ∼2.0 ns (75%) and 3.4 ns (25%).22 The fluorescence behavior is more complex in water than in other solvents: Balter et al.22 observed an additional, weak emission band around 430 nm. They also reported a biexponential fluorescence lifetime (τ1 ∼0.5 ns and τ2 ∼1.9 ns) with an additional small contribution from a long-lived component of ∼13 ns at the blue edge of the emission spectrum, exciting at 337 nm. The faster lifetime component in protic solvent was interpreted in terms of a solute–solvent complex formation via hydrogen bonding. Later, Bunker et al.29 noticed a stronger blue emission band exciting at 280 nm, and they suspected that the blue emission was the consequence of water-soluble impurities present in commercial PRODAN. Sun and co-workers41 resolved this ambiguity. They showed that the extra blue-edge shoulder and small contribution from the additional long-lived component (∼13 ns) in the fluorescence decay were the result of PRODAN–PRODAN intermolecular interactions in a supersaturated solution of PRODAN in water.

Our work here is motivated by previous studies using PRODAN to study the environments of reverse micelles.40,42–48 While most of these studies attributed PRODAN fluorescence to only the LE state and rationalized the observed emission to partitioning of the fluorophore in different regions of the reverse micelle,42–47 Novaira et al.40,48 have reported dual fluorescence emission band exciting at 280 nm, and they suspected that the blue emission was the consequence of water-soluble impurities present in commercial PRODAN. Solvent complex formation via hydrogen bonding. Later, Bunker et al.29 noticed a stronger blue emission band around 430 nm. They also reported a biexponential fluorescence lifetime (τ1 ∼0.5 ns and τ2 ∼1.9 ns) with an additional small contribution from a long-lived component of ∼13 ns at the blue edge of the emission spectrum, exciting at 337 nm. The faster lifetime component in protic solvent was interpreted in terms of a solute–solvent complex formation via hydrogen bonding. Later, Bunker et al.29 noticed a stronger blue emission band exciting at 280 nm, and they suspected that the blue emission was the consequence of water-soluble impurities present in commercial PRODAN. Sun and co-workers41 resolved this ambiguity. They showed that the extra blue-edge shoulder and small contribution from the additional long-lived component (∼13 ns) in the fluorescence decay were the result of PRODAN–PRODAN intermolecular interactions in a supersaturated solution of PRODAN in water.

The coexistence of different excited states with different properties of the same chromophores could have significant consequences for the accurate characterization of solvation dynamics in a heterogeneous environment, such as a protein. Therefore, the purpose of this work is to study the contributions of LE and CT states of PRODAN to its solvation dynamics, in particular, in reverse micelles formed by sodium 1,4-bis-(2-ethylhexyl) sulfosuccinate (AOT)/n-heptane/water (Figure 3). We have found that the LE and CT states of PRODAN solvate on different time scales and concluded that PRODAN’s use as a probe of heterogeneous environments must be used with caution.

Experimental Section

Materials. 6-Propionyl-2-(N,N-dimethylamino)naphthalene (PRODAN) (purity ≥98%) and sodium 1,4-bis-(2-ethylhexyl) sulfosuccinate (AOT) (purity >99%) were obtained from Sigma-Aldrich and used as received. n-Heptane was obtained from Acros, Spectragrade. Methanol and acetonitrile (purity 99.9%) were purchased from Fisher Scientific and used without further purification.

Sample Preparation. Stock solutions of PRODAN (0.05 M) in acetonitrile and AOT (0.8 M) in n-heptane were prepared. The reverse micelle system was prepared through volumetric dilution from the stock solutions. The final AOT concentration was 0.2 M. The concentrated PRODAN in acetonitrile solution was used to introduce the probe into the system. The final concentration of PRODAN was 5 × 10⁻⁵ M with an organic content of <0.1%. An appropriate amount of nanopure water was then added to obtain w = 20 (w = [H₂O]/[AOT]). All samples were allowed to equilibrate for 24 h before subsequent steady-state and time-resolved measurements. To prepare a PRODAN/water solution, 45 μL of a solution of 1 × 10⁻³ M PRODAN in methanol (Fisher Scientific, HPLC) was added to 5 mL of water; i.e., 9 × 10⁻⁶ M PRODAN/water was prepared with an organic content <0.9%. This freshly prepared solution was then used for the steady-state and time-resolved measurements.

Steady-State Measurements. Steady-state absorption spectra were obtained on a Hewlett-Packard 8453 UV–visible spectrophotometer with 1 nm resolution. Steady-state emission spectra were obtained on a Spex Fluoromax-4 with a 3 or 4 nm bandpass and corrected for lamp spectral intensity and detector response. For absorption and emission measurements, 5 mm and 1 cm path-length quartz cuvettes were used, respectively. All experiments were done at room temperature.

Time-Resolved Measurements. Measurements of excited-state lifetimes were performed with the time-correlated single-photon counting (TCSPC) technique. The apparatus for time-correlated single-photon counting is described elsewhere.39 The fundamental from a homemade mode-locked Ti:sapphire oscillator was modulated by a Pockels cell (model 350-160, Conoptics Inc.) to reduce the repetition rate to 8.8 MHz. All experiments were performed using either the 407 or 266 nm excitation obtained from the 814 nm fundamental by means of a U-Oplaz Technologies (Model TP-2000B) doubler/tripler. Recent modifications in the experimental setup include the replacement of NIM-style electronics by a Becker & Hickl photon counting module (model SPC-630). With this modified system, the full width at half-maximum of the instrument-response function is ~40–50 ps. A cuvette of 5 mm or 1 cm path length was used for the time-resolved measurement depending upon the system.

To construct the time-resolved spectra, a series of decays were collected typically from 370 to 560 nm at 10 nm intervals. Transients were fit to sums of exponentials, and time-dependent spectra were reconstructed from these fits by normalizing to the steady-state spectra:
\[ S(\lambda, t) = D(\lambda, t) \frac{S_0(\lambda)}{\int_0^\infty D(\lambda, t) \, d\lambda} \]  

\( D(\lambda, t) \) is the wavelength-resolved fluorescence decay, and \( S_0(\lambda) \) is the steady-state emission intensity at a given wavelength. We have employed the traditional approach of fitting the time-resolved spectra to a log-normal function, from which we extract the peak frequency, \( \nu(t) \), as a function of time.

Results and Discussion

The absorption and emission spectra of PRODAN in AOT/n-heptane/water (\( w = 20 \)) are shown in Figure 4. The emission spectrum of PRODAN in AOT/n-heptane/water exciting at 266 nm consists of two bands. This result is consistent with the dual emission obtained previously exciting at 330 nm in AOT/n-heptane/water reverse micelles from a locally excited state (LE) at \( \lambda_{\text{em}}^{\text{LE}} = 411 \) nm and from a charge-transferred state (CT) at \( \lambda_{\text{em}}^{\text{CT}} = 507 \) nm. Upon excitation at 407 nm, only one emission band was observed, with \( \lambda_{\text{em}}^{\text{CT}} = 508 \) nm. This indicates that only PRODAN molecules in the polar environment of AOT/n-heptane/water reverse micelles are excited at red-edge excitation. Thus, it is possible to observe PRODAN emission from both the LE and CT states in the same sample, and its emission can be tuned with excitation energy.

Fluorescence lifetime decays of PRODAN in water, n-heptane, and AOT/n-heptane/water (\( w = 20 \)) at different excitation wavelengths are displayed in Figure 5. Figure 5A presents the decay traces of PRODAN in n-heptane and water exciting at 266 nm. The lifetime parameters are given in Table 1. PRODAN has a single-exponential lifetime with a time constant of 0.15 ns in n-heptane. The decay of PRODAN in water is well described by a biexponential function with time constants of 0.70 ns (60%) and 2.0 ns (40%). There is a significant change in the decay kinetics of PRODAN in AOT/n-heptane/water (\( w = 20 \)) depending on whether the excitation wavelength is 266 or 407 nm (Figure 5B). The trace obtained with 266 nm excitation was best fit with a biexponential function with time constants of 0.16 ns (70%) and 2.3 ns (30%). The trace obtained with 407 nm, however, was best fit by a single exponential with a time constant of 2.6 ns. The absence of a risetime of the CT emission band (Table 1, Figure 5B) suggests that in such a polar environment the LE \( \rightarrow \) CT reaction rate of PRODAN is too fast to be resolved under the present experimental conditions. 4-N,N-dimethylaminobenzonitrile (DMABN) is a well studied molecule which emits from both LE and CT states and shows dual emission bands in polar solvents. It is important to note that DMABN also leads to a complete cutoff of the LE emission band and shows only the CT emission band upon red-edge excitation of its absorption spectrum in polar solvent. The charge-transferred time found for DMABN in AOT/n-heptane is important to note that DMABN also leads to a complete cutoff of the LE emission band and shows only the CT emission band upon red-edge excitation of its absorption spectrum in polar solvent. The charge-transferred time found for DMABN in AOT/n-heptane is estimated to be 4–6 ps. To investigate the solvation of the LE and CT states, wavelength-resolved lifetime measurements were carried out at 20 different wavelengths from 370 to 560 nm for PRODAN in AOT/n-heptane/water (\( w = 20 \)) and were fit to a sum of two decaying exponentials. Representative wavelength-resolved fluorescence decay traces are displayed in Figure 6. To evaluate the spectra and dynamics of the LE and CT states separately, the emission spectrum in Figure 4 was decomposed into two bands using log-normal functions. Time-resolved emission spectra were constructed according to eq 1 for each using the fitting parameters for the 20 wavelength-resolved decay traces. Representative time-resolved emission spectra for the LE and CT states are given in Figure 7. Plots of peak frequencies as a function of time are given in Figure 8 for the LE and CT states. The peak shift was fit to a single exponential of time constant 2.0 ns for LE and to two exponentials with time constants of 0.40 ns (91%) and 4.0 ns (9%) for CT.

These results clearly demonstrate that the solvatochromic probe, PRODAN, can emit from different states in a heterogeneous environment and that, owing to the different natures of these states (i.e., charge-transferred or not), their solvation dynamics can occur on different time scales and they can have significantly different Stokes shifts. This, consequently, introduces another level of complication into the interpretation of...
the data when fluorescence is observed from chromophores capable of emitting from more than one excited state. If the properties of each state are known, it is possible to take them into account. We have recently discussed and compared two methods of performing such an analysis.55

There are other factors, however, that are more difficult to account for, namely, the effects of slow conformational changes. We have recently discussed and compared two methods of performing such an analysis.55

or aggregation, which is possible at the high concentrations (~1 mM)56,57 at which some of these experiments are performed. We now refer to a few examples. As illustrated in Figure 1, more elaborate fluorescent probes of biological systems are based upon the PRODAN chromophore, namely, DANCA58 and, more recently, a nonnatural amino acid analogue, Aladan.59 Boxer and co-workers56 have incorporated the latter at different buried and exposed sites of the immunoglobulin binding domain, B1, of protein G (GB1) and measured the time-dependent Stokes shift. All of the sites showed a bimodal relaxation with an inertial ultrafast response of ~80–140 fs followed by a much slower relaxation on the time scale of several picoseconds to several nanoseconds, depending upon the location of the probe. This experimental work forms the basis for a good comparison with simulations performed by Golosov and Karplus.60 In the context of their study, an interesting observation was that the time-resolved emission spectra of Aladan at fully and partially exposed sites of the protein showed a blue shift at long times (~1 ns). The authors suggested that this blue shift could be attributed either to different chromophore populations having different lifetimes and solvation dynamics or to aggregation effects.

On the basis of our above results for the PRODAN photophysics and solvation dynamics, we suggest a model that can produce a blue shift of time-resolved spectra at long times. In this model, we assume that the LE state is formed from CT states. In particular, we assume a slow ground-state conformational change or aggregation that perturbs the environment of the chromophore in such a manner that CT states are no longer predominantly favored and thus increases the population of LE states. As LE is gradually formed in this manner, it will undergo its characteristic solvation dynamics, which must be convoluted with the LE formation and decay in order to obtain the complete spectral response of this newly formed species. The spectrum at any given time will then have contributions from both CT and LE.

Thus, the time course of production and decay of LE, \( L(t) \), is given by

\[
L(t) = I_0(e^{-\frac{t}{\tau_1}} - e^{-\frac{t}{\tau_{LE}}})
\]

where \( I_0 \) is the fraction of the protein population that undergoes the conformational change discriminating against the formation of CT and instead favoring the production of LE. In our simulation, we took \( I_0 = 0.1 \) (~20% of the CT population, as given by the spectral decomposition of eq 1). The time of formation, \( \tau_f \), of LE from CT was chosen as 5.0 ns. The fluorescence lifetime of LE, \( \tau_{LE} \), was found experimentally to be 0.15 ns. The evolution of time-resolved spectra of LE is

\[
I(\lambda, t) = A e^{-\frac{1}{2}(\lambda - \lambda_{t=0})^2/\sigma^2} e^{-\frac{t}{\tau_{LE}}}
\]

where \( A = 2.4 \) and was obtained by spectral decomposition using eq 1 and \( \sigma = 1280 \text{ cm}^{-1} \), as obtained from the time-resolved emission spectrum at the instrumental time zero. The peak frequency of the time-resolved spectra is given by \( \lambda_m = (\lambda_0 - \lambda_\infty)e^{-\frac{t}{\tau_{t=0}}} + \lambda_\infty \), where \( \lambda_0 \) and \( \lambda_\infty \) were taken from the experimental data as 24675 and 20292 cm\(^{-1}\), respectively. The solvation time, \( \tau_{sol} \), of LE is 2.0 ns (Figure 8). The convolution of \( L(t) \) with \( I(\lambda, t) \) is

\[
LE(\lambda, t) = \int_0^t L(t')I(\lambda, t - t') \, dt'
\]
and yields the time-resolved emission spectrum induced by the slow conformational change or aggregation postulated above (Figure 9). The time-resolved spectra for LE and CT were then summed to generate the total time-resolved spectra. Those at 0, 1.0, and 10.0 ns are shown in Figure 10. At 0 ns, there is virtually no contribution from LE in the total time-resolved spectrum, but with increasing time, LE contributes significantly to the total time-resolved spectrum. Other variations of this model are clearly possible.

Given, however, that the LE state was not accessed with 400 nm excitation (Table 1), the excitation wavelength used by Boxer and co-workers, and that their steady-state spectra showed no evidence of the LE state, the blue shift they observed is likely to have other origins. On the other hand, it is useful to consider three different investigations carried out to study the solvation response of myoglobin using various chromophores to replace the heme. These chromophores are the PRODAN derivative, DANCA, another charge-transfer molecule capable of emitting from dual states, aniline-2-aminoanthalene-6-dimethylsulfonamide (ANSDMA), and coumarin 153. Each probe of solvation dynamics exhibited a different response. DANCA yielded a complicated solvation response with significant contributions into the nanosecond regime. ANSDMA provided a single-exponential response of ~9 ns (although rapid components might have been neglected because of the time resolution of the experiment). Using coumarin 153, it was found that almost 60% of the solvation is complete within the time resolution of the experiment (300 fs) and that this initial response is followed by a slower one. Most importantly, there was excellent agreement between the solvation correlation function, \( C(t) \), from fluorescence upconversion experiments and those obtained from molecular dynamics simulations. While some of these differences may be attributed to how or whether a solvation correlation function was actually constructed, it is legitimate to inquire whether some of the discrepancy might not also be attributed to the nature of the fluorescent probes employed. While DANCA and ANSDMA are both capable of charge transfer in the excited state, coumarin 153 is exquisitely inert, which is one of the reasons it has been so extensively employed as a probe of solvation. (The DANCA experiments used an excitation wavelength of 345 nm. Novaira et al. have observed that both LE and CT emission can be observed in reverse micelles at 330 nm. While the DANCA work does not mention a blue-shift at long times, it is feasible that some of the long-time dynamics observed are a result of the possible behavior we have suggested above.)

**Conclusions**

Stimulated by the work of Novaira et al., we have studied the solvation dynamics of PRODAN in a heterogeneous environment (reverse micelles) that permits a distribution of the chromophores to emit from both the LE and CT states. We have studied the solvation dynamics of both states and found them to be very different, as suggested by the work of Novaira et al. This result consequently leads to the question of whether PRODAN-based chromophores or, more generally, any chromophore capable of undergoing excited-state photochemistry, can induce artifacts into the interpretation of solvation dynamics in heterogeneous environments, in particular, those provided by biological systems such as proteins. Considerable care in choosing and characterizing the system is required in order to analyze the results fully.

**References and Notes**
