Ground- and Excited-State Aggregation Properties of a Pyrene Derivative in Aqueous Media

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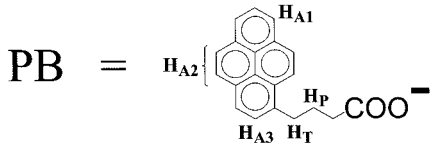
The aggregation properties of 4-(1-pyrene)butanoate in aqueous media have been quantitatively investigated. For the pyrene chromophore ground state, the monomer−dimer equilibrium was monitored using NMR methods, yielding a dimerization constant of 150 M^{-1} for pyrenebutanoate in alkaline water. In addition, the excited-state aggregation was studied by time-resolved emission spectroscopy. The excimer formation constant in water, which represents the equilibrium established by interaction of excited- and ground-state species, was determined to be 1.6×10^4 M^{-1} (about 100-fold larger than the ground-state dimerization constant). The role of solvent polarity and the influence of an aromatic cosolvent were examined in order to determine the types of interactions that contribute to the driving force for aggregation. The ground-state aggregation propensity of pyrenebutanoate was significantly diminished upon addition of methanol or pyridine to aqueous solutions, indicating that hydrophobic and/or π-stacking interactions play a role in the aggregation processes. Similar trends were observed also in the case of excited-state aggregation when the organic cosolvents were included. The investigation provides a quantitative assessment of the thermodynamics of interaction of pyrene fluorescence probes that are widely used in aqueous media in biophysical studies.

Alkylpyrene derivatives have been widely used to probe macromolecular conformations utilizing the emission properties of the monomeric and aggregated aromatic chromophore.[1−3] Upon excitation in the UV region, monomeric pyrene exhibits fluorescence at 370−430 nm with well-resolved vibronic features. However, if another chromophore is in proximity, the monomer fluorescence is diminished in intensity and a broad featureless emission band peaking at about 485 nm appears.[3] The so-called "excimer fluorescence" has been observed for pyrene solutions at high chromophore concentration,[4−7] for pyrenes incorporated in micelles,[3,8] when chromophores are linked together via short chains,[3,9] or when macromolecular quaternary structures bring two chromophores into proximity.[1,2] A concern in using pyrene derivatives as aggregation fluorescence probes has to do with the erroneous assumption that they are "passive probes". In fact, derivatization with pyrene increases the aggregation propensity of macromolecules frequently leading to structural alterations (e.g., in the formation of peptide helix "bundles").[10,11] The purpose of this publication is to provide a quantitative treatment of alkylpyrene aggregation properties in aqueous environment in order to fractionate the association free energies of chromophore tagged macromolecular species, i.e., to determine the portion of the total aggregation energy that is a result of chromophore−chromophore interactions in labeled macromolecules.[12] To better understand the driving factors that influence aggregation, the effects of introduction of relatively less polar and aromatic cosolvents was examined. The free energies of formation of ground- and excited-state aggregates were extracted from NMR and dynamic fluorescence data.

Experimental Section

Materials. 4-(1-Pyrene)butanoic acid was purchased from Aldrich and doubly recrystallized from ethanol. The solvents (HPLC and spectroscopic grade) were purchased from VWR.

SCHEME 1: Structure of 4-(1-pyrene)butanoate (PB)



Milli-Q water was used for all experiments. The deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc.

The 4-(1-pyrene)butanoate samples were prepared by dispersing small volumes of concentrated DMSO (or DMSO-d_6) solutions of pyrenebutanoic acid in the appropriate alkaline aqueous solvent. The samples for the emission experiments were purged with argon for about 30 min prior to the measurements.

Methods. The NMR spectra were recorded using a 400 MHz Varian spectrometer. The UV/vis absorption measurements were conducted on a Beckman DU 640B spectrophotometer. The emission spectra were recorded using a PTI Fluorescence System with FeliX software. The reported stroboscopic lifetime measurements in this study were performed on PTI TimeMaster Fluorescence Lifetime Spectrometer, equipped with a PTI GL-3300 nitrogen laser for excitation at 337 nm.

Results and Discussion

Ground-State Aggregation. An approach using ^{1}H NMR spectroscopy[13,14] was employed to examine the behavior of D_2O solutions of 4-(1-pyrene)butanoate (PB, see Scheme 1) at various concentrations. The NMR spectra obtained for samples covering the low millimolar concentration range are presented in Figure 1. A very significant upfield signal shift is observed with increasing concentration, which can be assigned to the formation of molecular aggregates. The shifts of three aromatic and two

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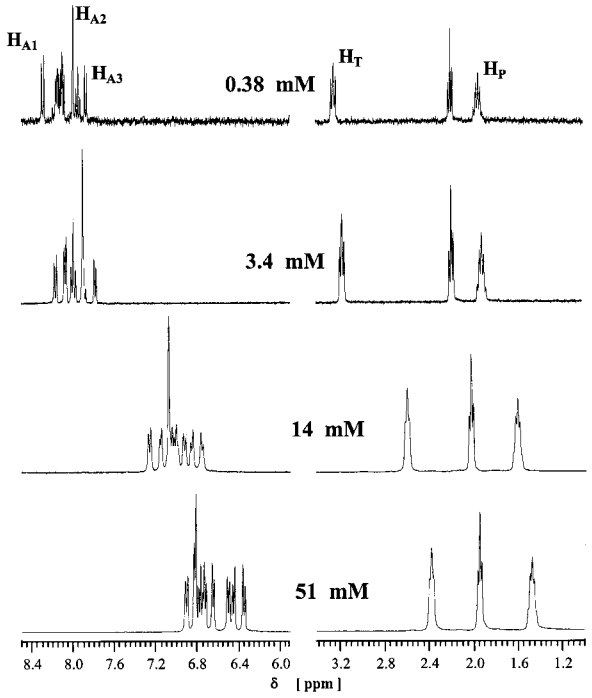


Figure 1. One-dimensional proton NMR spectra for PB at various concentrations in D_2O (+ 2% NaOD).

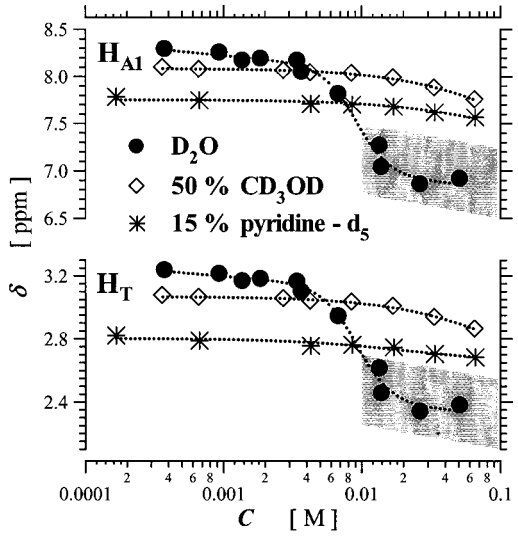


Figure 2. Examples of alteration of the NMR chemical shifts in the aromatic (H_{A1}) and aliphatic (H_T) regions. (The shaded area represents the meta-stable solution: i.e., a concentration range where precipitate formed in an 8−24 h period.)

aliphatic protons (noted on Scheme 1 and Figure 1) were followed in their titration-like behavior (Figure 2).

An assumption for one-step aggregation (i.e., nPB $\rightleftarrows$ $(PB)_n$),[13−15] yields the following expressions for the total PB concentration, C, and the aggregation equilibrium constant, K:

$$C = [PB] + [(PB)_n] \tag{1}$$

$$K = \frac{[(PB)_n]}{[PB]^n} \tag{2}$$

where [PB] and $[(PB)_n]$ are the equilibrium concentrations of the monomer, PB, and the aggregate of n monomeric units, $(PB)_n$, respectively.

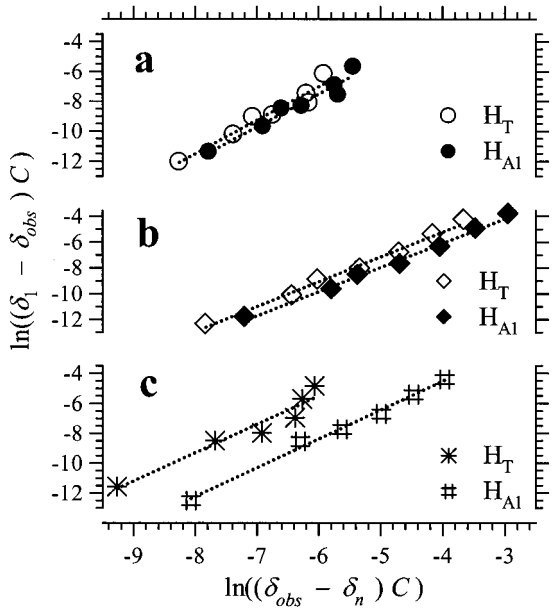


Figure 3. Treatment of NMR shifts for PB in various solvents using the logarithmic function described (eq 5): (a) D_2O; (b) 50% (v) CD_3OD in D_2O; and (c) 15% (v) pyridine-d_5 in D_2O.

Assuming that the aggregation kinetics is much faster than the NMR data collection time,[13,14] the observed shift of each proton, δ_{obs}, can be expressed as a weighted sum of all the shifts it will exhibit for the distinct inter-exchangeable species, $\delta_{obs} = 1/C \sum_i C_i\delta_i$, yielding the following expression for an one-step aggregation equilibrium:

$$\delta_{obs} = \frac{[PB]}{C}\delta_1 + \frac{n[(PB)_n]}{C}\delta_n \tag{3}$$

where δ_{obs} is the observed NMR shift, δ_1 is the shift of the same proton for the PB monomer, and δ_n for the PB aggregate. The values for the latter two, δ_1 and δ_n, were obtained by extrapolation of the shift data (Figure 2) to concentrations zero and infinity, respectively.

Combining eqs 1 to 3, followed by proper rearrangement, yields the following relation for the aggregation parameters and the NMR shifts:

$$(\delta_1 - \delta_{obs})C = n\frac{(\delta_{obs} - \delta_n)^n}{(\delta_1 - \delta_n)^{n-1}}KC^n \tag{4}$$

that can be transformed into a linear dependence by taking the natural logarithm for both sides of the equation:

$$\ln((\delta_1 - \delta_{obs})C) = n\ln((\delta_{obs} - \delta_n)C) + \ln(K) + \ln(n) + (1-n)\ln(\delta_1 - \delta_n) \tag{5}$$

Equation 5 was applied to the five NMR signals whose shifts were followed; the slope of the plots yielded the state of aggregation, n, while the intercept provided the aggregation equilibrium constant, K (Figure 3a).[14] The results suggest that the PB aggregation for the observed concentration range proceeds to a dimer, $n = 2.14 \pm 0.06$, with dimerization constant, $K = 150\ M^{-1}$ ($\ln(K) = 5.0 \pm 0.5$), a value which is similar to the results reported for another pyrene derivative.[14] Applying the relation $\Delta G = -RT\ln(K)$, the free energy of

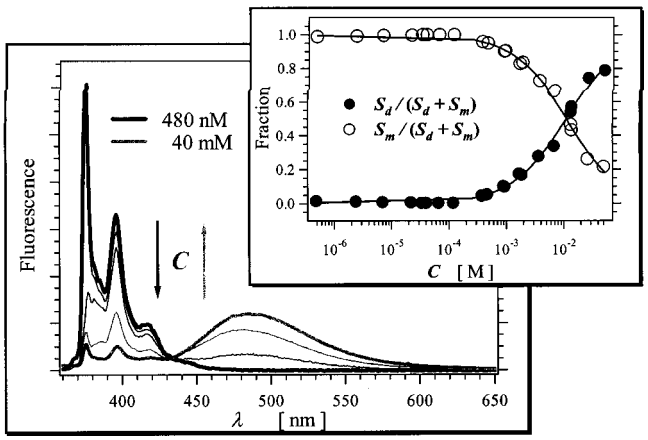
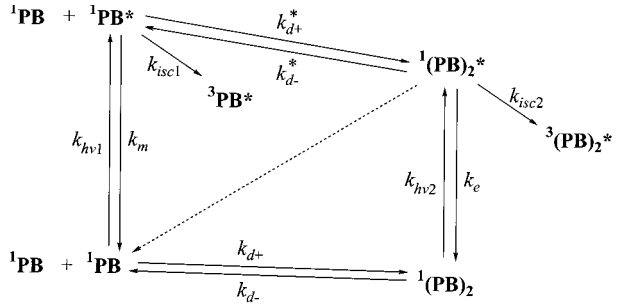


Figure 4. Emission spectra of PB (various concentrations) in water + 1.5% NaOH (λ_{ex} = 333 nm). S_m and S_d represent the areas under the monomer and dimer fluorescence spectra, respectively: $S = \int F(\upsilon)\, d\upsilon$.

SCHEME 2: Kinetics of Ground- and Excited-State Aggregation of PB Chromophore



formation of the PB ground-state dimer in water was calculated ($\Delta G_{20°C}$ = −2.9 kcal/mol = − 0.13 eV).

Excited-State Aggregation. The excited-state association (i.e., excimer formation) has been extensively studied for pyrene derivatives.[3−8] However, few of the previous investigations pertained to the aqueous solvent media that are most relevant to polypeptide and protein studies. Attempts to generate PB excimers have been reported;[2,10] however, the concentrations attained were not high enough for excimer generation.[16]

The fluorescence spectra of PB in water at various concentrations (Figure 4) demonstrate that above ∼1 mM the monomer emission (379−430 nm) is quenched with concurrent growth of a broad featureless excimer band at 485 nm. At these high concentrations the excited dimer can be obtained either (1) by direct photoexcitation of the ground-state dimer, or (2) by aggregation of an excited- and ground-state chromophores (i.e., excimer formation—see k_{hv2} and k^*_{d+}, Scheme 2). The emphasis of this section is on the latter process.

For investigation of excited-state kinetics, time-resolved fluorescence spectroscopy was applied. Pyrene systems of this type have been extensively studied and the required mathematical treatment has been previously developed in detail by Birks et al.[6] However, in previous studies the ground-state aggregation has not been taken into consideration.[6,7] Therefore, the Birks approach is applicable in this investigation with the following limitations:[17] (1) the total PB concentration is to be kept relatively low (0.5 mM and less) in order to prevent excimer formation from direct ground-state dimer excitation; (2) use of low excitation pulse energies is necessary to ensure that the concentration of the excited-state species is negligible compared to the total PB concentration through the whole time range (i.e., [PB] ≈ C = *constant*); (3) based on the previous two conditions for low PB concentrations, at any time [$(PB)_2$] ≪ [PB], thus, the excimer decay can be presented to proceed directly to the ground-state monomer (see the dashed arrow in Scheme 2); (4) since the time span of the measurements is kept below 1 μs, all the triplet pathways leading to regeneration of the singlet excited state and delayed fluorescence[5] can be neglected and the excited-singlet-to-triplet intersystem crossing rate constants are included in the decay rate constants, k_m and k_e.

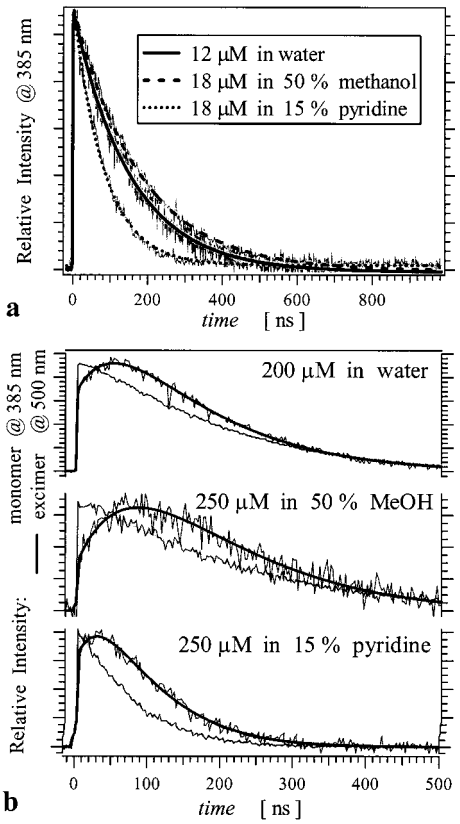


Figure 5. Emission decays of PB in aqueous solvents in the presence of 1% NaOH (λ_{ex} = 337 nm): (a) for relatively low PB concentrations at which excimer formation is not observed; and (b) for relatively high PB concentrations, illustrating decay of monomer emission and growth and decay of excimer emission (see text, eqs 6 and 7).

It has been reported that upon short nanosecond pulse excitation of millimolar pyrene solutions, the monomer fluorescence spectrum with traces of excimer fluorescence appears with the pulse. Consequently, the monomer fluorescence disappears gradually in tens of nanoseconds, and concurrently the intensity of the excimer emission increases, goes through a maximum, and then decays.[7] The same behavior was observed in the case of aqueous PB solutions (Figure 5). Adopting the annotations introduced by Birks, $X = k_m + k^*_{d+}\, C$ and $Y = k_e + k^*_{d-}$, the monomer and the excimer fluorescence decays can be represented by the following biexponential time dependent functions:[6]

$$F_m = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t} \tag{6}$$

$$F_e = D(e^{-\lambda_1 t} - e^{-\lambda_2 t}) \tag{7}$$

where,

$$\lambda_{1,2} = \frac{1}{2}\left(X + Y \mp \sqrt{(Y-X)^2 + 4k_{d+}k_{d-}C}\right) \tag{8}$$

TABLE 1: Photophysical Data and Aggregation Constants for PB in Alkaline Water and Water with Organic Cosolvents

solvent	Φ_f[a]	τ_f[b] [ns]	k_d^{-1}[c] [ns]	$k^*_{d+} \times 10^{-9}$[c] [M^{-1} s^{-1}]	$k^*_{d-} \times 10^{-6}$[c] [s^{-1}]	K* [M^{-1}][d] ($-\Delta G^*$ [eV])[f]	K [M^{-1}][e] ($-\Delta G$ [eV])[f]
water	0.64	153	132	33.	2.1	1.6×10^4 (0.20)	150 (0.10)
50% MeOH	0.53	176	199	1.3	2.9	450 (0.13)	2.5 (0.019)
15% pyridine	0.24	81.1	63	22.	2.1	1.0×10^4 (0.19)	7.5 (0.042)

[a] From steady-state emission data (see Figure 7). [b] From single-exponential fits of time-resolved emission data for PB concentrations smaller than 20 μM. [c] From time-resolved emission data for PB concentrations between 100 and 500 μM (see text for details). [d] $K^* = k^*_{d+}/k^*_{d-}$. [e] From NMR data (see text for details). [f] Calculated for 20 °C.

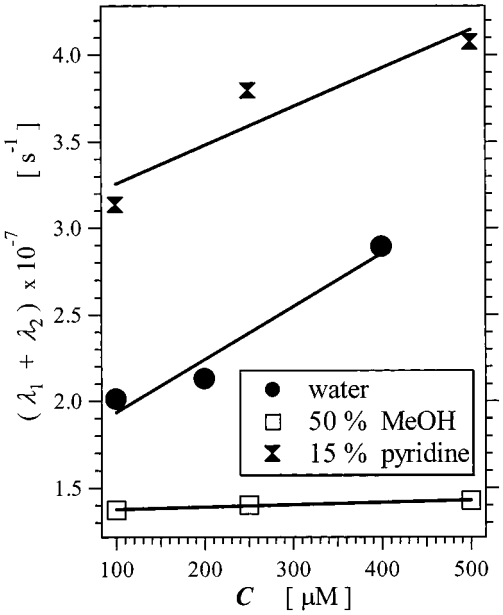


Figure 6. Analysis of the sums of the decay constants, λ_1 and λ_2 (see eq 9).

Apparently, the sum of the rate factors, λ_1 and λ_2, depends linearly on the total chromophore concentration, C:

$$\lambda_1 + \lambda_2 = k_m + k_e + k_{d-} + k_{d+}C \tag{9}$$

also,

$$k_{d-} = \frac{(X - \lambda_1)(\lambda_2 - X)}{k_{d+}C} \tag{10}$$

and

$$k_e = Y - k_{d-} \tag{11}$$

Experimentally, the monomer decay constant, k_m, was extracted from a single-exponential fit of fluorescence data for 12 μM PB in water (Figure 5a). Consequently, the rate factors, λ_1 and λ_2, were measured at various higher concentrations (Figure 5b) and their sums vs C were fit to a line (Figure 6). The value of the slope of the fit was ascribed to the excimer formation rate constant, k^*_{d+}, and the rate factor Y, was extracted from the difference between the intercept and k_m (see eq 9). The other two rate constants, k_e and k^*_{d-}, were calculated using eqs 10 and 11. The excimer formation equilibrium constant, K^*, can be represented as a ratio between the forward and backward rate constants, i.e., $K^* = k^*_{d+}/k^*_{d-}$. Having a value of 1.6×10^4 M^{-1} (Table 1), K^* for PB in water is similar to the previously reported excimer formation constant for pyrene in ethanol, $\sim 1.0 \times 10^4$ M^{-1} at room temperature.[6] Furthermore, the corresponding free energy of PB excimer formation was calculated to be -5.6 kcal/mol (-0.24 eV), which is 2.7 kcal/mol more negative than the ground-state dimerization free energy. This higher excimer stability (of about 0.1 eV) is not surprising since it supports the widely accepted understanding of the nature of excimer formation.[18] The additional important feature for the PB aqueous systems reported in this publication is the formation of a stable ground-state dimer observed at higher chromophore concentrations.

Influence of Organic Cosolvents. To examine the role of hydrophobic and π-electron interactions in PB aggregation, experiments were conducted using less polar and aromatic cosolvents,[19] methano,l[20] and pyridine,[21] respectively. For ground-state aggregation, the NMR signals were shifted upfield upon increase in the chromophore concentration (Figure 2), similar to the behavior of the solutions in pure water. However, when organic cosolvents were present, the changes occurred at higher PB concentrations and, in the case of 15% (v) pyridine, they were not as large. The states of aggregation and the corresponding aggregation constants were extracted by applying eq 5 to the data (Figure 3b and 3c). In both cases the slopes of the linear fits suggested that PB for the concentration range examined aggregates to dimer. The results obtained for the dimerization constants were 2.5 M^{-1} for 50% (v) methanol, and 7.5 M^{-1} for 15% (v) pyridine, i.e., values that are, respectively, 60 and 20 times smaller than the value obtained for 100% water. This dramatic decrease of the propensity for PB aggregation upon addition of a less polar solvent, methanol, is a clear indication that the intermolecular hydrophobic interactions are a major driving force in the dimerization process.

A more interesting result is that the addition of only 15% aromatic cosolvent shows also a significant decrease in the size of the aggregation constant. A plausible explanation is that the π-electron-rich pyridine interacts with the π-electron system of the chromophore resulting in displacement of the pyrene molecules away from each other. This effect would explain why the NMR signals at low PB concentrations in the presence of pyridine are considerably upfield shifted (0.5 to 1 ppm compared to 100% D_2O solutions), and why pyrene−pyrene aggregation, observed in the higher concentration range in 15% (v) pyridine, does not cause as much deshielding as in 100% water or even in 50% (v) methanol (Figure 2). Further evidence for pyrene−pyridine interaction and perturbation of the pyrene π-electron system can be found in the PB UV/vis absorption and emission spectra in the three different solvent media (Figure 7). While there is no significant difference in spectral data between the water and the 50% (v) methanol solutions, when 15% (v) pyridine is present, (1) the absorbance and emission bands are shifted to the red about 2 to 4 nm; (2) a slight decrease of the extinction coefficient is detected; and (3) the fluorescence quantum yield (at low concentrations), together with the excited monomer and excimer lifetimes, is decreased two-to-three times (Table 1).

Similar trends were observed in the excited-state aggregation properties. For 50% (v) methanol solution, the finding of an excimer formation constant of 450 M^{-1}, 36 times smaller than

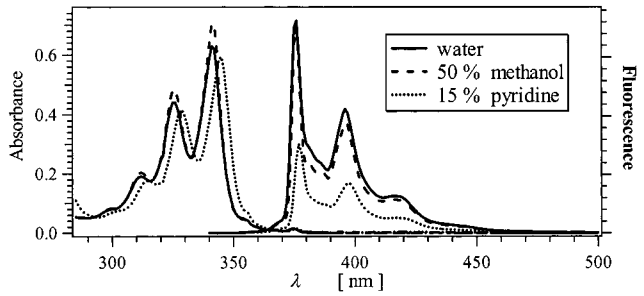


Figure 7. Absorption and fluorescence spectra for 21 μM PB in the presence of 1 mM phosphate buffer, pH 8, in various solvent mixtures ($\lambda_{ex} = 333$ nm).

the value measured in water, indicates that the hydrophobic intermolecular interactions also make a significant contribution to the excited state aggregation. However, for PB in 15% (v) pyridine, the measured excimer formation constant was only 1.6 times smaller ($K^* = 1.0 \times 10^4$ M^{-1}) suggesting that, in addition to the higher excimer stability, the pyridine interaction with the excited state alkylpyrene chromophore is considerably weaker. A summary of the photophysical and the aggregation constants of pyrenebutanoate in aqueous media is presented in Table 1. It is quite noticeable that the solvent does not have a very strong effect on the excimer dissociation rate constants, k^*_{d-}. However, the excimer formation rate constants, k^*_{d+}, change by more than an order of magnitude upon altering the solvent composition, suggesting that specific solute−solvent interactions have a significant effect on the activation energy for the bimolecular association. Thus, the thermodynamic changes observed upon solvent variation, can be ascribed predominantly to the interactions of the monomeric alkylpyrene species with the surrounding medium.

Conclusions

4-(1-Pyrene)butanoate has a significant propensity for aggregation not only when excited, but also in the ground state. In water, the process of chromophore dimerization has a thermodynamic driving force for the ground-state, $\Delta G = -0.13$ eV; for "excimer" formation, $\Delta G^* = -0.24$ eV. Therefore, caution should be applied when pyrene derivatives are used as fluorescence probes to study quantitatively the energy of self-assembly for more complicated (supramolecular) systems. Indeed, an alkylpyrene will contribute an additional 3 kcal/mol ($\Delta\Delta G = 0.13$ eV) of stabilization energy (in aqueous solutions), leading to orders of magnitude change in a macromolecular aggregation constant[11] and even an increase in the state of aggregation.[10] The importance of the polarity of the media in adjusting the propensity for aggregation has also been shown. Upon addition of 50% of methanol to mixed aqueous solutions, the free energies of dimerization are 0.08 and 0.07 eV more positive for the ground- and the excited-state processes, respectively, representing shift of equilibria toward the monomeric species. The finding of a role for solvent polarity emphasizes the concern over possibly misleading results, especially quantitative ones, obtained in biophysical fluorescence probe experiments. The majority of these measurements are performed in buffered aqueous solutions[1,2] and cannot be directly correlated to the extensive quantitative work on pyrene performed in ethanol and other organic solvents.[4,6,7]

The role of an aromatic cosolvent, pyridine, in the ground-state PB aggregation was significant, resulting in a dimerization free energy more positive by about 0.08 eV, emphasizing the importance of the associative properties of π-electron systems for other organic chromophores used as fluorescence probes.[10]

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(15) Significantly more complicated, multiple-step aggregation models were also applied; however, they did not yield reliable data fits.

(16) To solubilize pyrene derivatives in aqueous media, charged species are required. Therefore, solutions of pH 8 and higher were used to make sure that the butanoate is completely deprotonated. The presence of DMSO causes precipitation of concentrated ionic compounds. Thus, the DMSO concentration was kept under 4% for aqueous samples.

(17) To find an explicit expression for the time dependence of the ground- and excited-state monomer and dimer concentrations that can be used to analyze the emission data in the full concentration range, it is essential to solve the following multidimensional nonlinear system of differential equations: $d\mathbf{x}/dt = \mathbf{Ax} + \mathbf{F}$. If the contribution from the triplet-excited states is neglected, for instance, the three matrixes have the following form:

$$\mathbf{x} = \begin{pmatrix} [PB^*] \\ [(PB)_2^*] \\ [PB] \\ [(PB)_2] \end{pmatrix}, \qquad \mathbf{A} = \begin{pmatrix} -k_m & k^*_{d-} & k_{hv1} & 0 \\ 0 & -\left(k_e + k^*_{d-}\right) & 0 & k_{hv2} \\ k_m & k^*_{d-} & -k_{hv1} & k_{d-} \\ 0 & k_e & 0 & -\left(k_{hv2} + k_{d-}\right) \end{pmatrix}, \qquad \text{and}$$

$$\mathbf{F} = \begin{pmatrix} -k^*_{d+}[PB^*]\,[PB] \\ k^*_{d+}[PB^*]\,[PB] \\ -\left(k^*_{d+}[PB^*]\,[PB] + k_{d+}[PB]^2\right) \\ k_{d+}[PB]^2 \end{pmatrix}.$$

For pulse excitation experiments there is no absorption during the decay: $k_{hv1} = k_{hv2} = 0$. In addition, for steady-state conditions the time derivatives have zero value and the system turns into $\mathbf{Ax} + \mathbf{F} = 0$. To find a solution of this system is quite a challenging task because of the nonlinear factor, **F**, and is beyond the scope of this work. Furthermore, should the delayed fluorescence be considered in the analysis of the steady-state data, the concentrations of the triplet−excited processes must be included.[5] Therefore, having made the reasonable approximations described, we adopted the method of Birks et al. that involves solving only two-dimensional autonomous linear system of differential equations.[6]

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