Journal of Fluorescence, Vol. 9, No. 3, 1999

Fluorescence Analysis of Acridine Orange Adsorbate at the Water/*N*-Heptane Interface, Bulk and Interface

M. M. Murad[1]

Received August 7, 1998; accepted February 9, 1999

The amphiphilic nature of 3, 6-bisdimethylaminoacridine (trivial name, acridine orange) can be used to characterize (polar/nonpolar) interfaces. This paper studies the properties of acridine orange and its dodecyl derivative at a solvent interface by internal reflection and tension determinations. In the case of absorbance measurements of crystallized acridine orange, the *potenz* value of the conventional acid dissociation constant pK_a was found to be 9.6. A clear isosbestic point could be obtained after purification at a unique pH for different concentrations. In contrast, in the absorbance measurements of acridine orange dodecyl, no isosbestic point was observed; this was attributed to the free long dodecyl chain. The overlap in the absorbance spectrum and the normalized fluorescence spectrum was found in many solvents around 500 nm. Neutral species were more likely to transfer into organic phase upon shaking. For fluorescence measurements both steady-state and time-resolved spectra were investigated. Results showed that at the interface a bathochromic shift happened to the maxima. This means that chromophore species aggregated at the interface as the dimer or trimer or the macro oligomer. Fluorescence polarization in bulk was calculated and found to be ca. 0.4. For interfacial measurements a dynamic volume method was applied to obtain the interfacial tension. An extrapolated Gibbs plot was obtained. Increasing bulk concentration increased the concentration at the interface to a finite value representing saturation.

KEY WORDS: Aggregation; fluorescence polarization; interfacial tension; liquid–liquid interface; total internal reflection fluorescence spectroscopy.

INTRODUCTION

Interest in the interface of two immiscible phases has increased recently because it has many applications in biology, pharmacy, microemulsions, and industry. Acridine compounds are important therapeutically. The affinity and nonaffinity to the interface represent adsorption and desorption, respectively. The strength of the formed/detached bonds influences this equilibrium. Interfaces have special properties that have fascinated and drawn the attention of scientists and engineers from many fields. What makes the interface unique is the asymmetry in forces experienced by molecules and species located at the interfacial layer with the three-dimensional geometry of the interface. Variables such as the chemical composition, geometrical arrangement of the species, hydrogen bond formation in protonic solvents, equilibrium constants, pH, motion of molecules, thermodynamics and kinetics of ground- and excited-state chemical change, energy relaxation, and phases and phase transitions of long-chain amphiphilic monolayers are among the fundamental manifestations of the unique characteristics of an interface.

Despite the importance and widespread interest, it remains very difficult to probe the chemistry and physics of interfaces, in particular, liquid interfaces using tradi-

[1] Chemistry Department, Faculty of Sciences, Tohoku University, Aramaki Aza Aoba, Aoba-ku, Sendai, ZIP 980-8578, Japan. Fax: (81)22-217-6550. e-mail: murad@anal.chem.tohoku.ac.jp

1053-0509/99/0900-0257$16.00/0

tional spectroscopic techniques. The reason is that the overwhelmingly large number of solute molecules in the bulk can interfere with the signal originating from solute molecules in the interface.

The characterization of liquid–liquid interfaces can be done mainly by thermodynamical techniques represented by interfacial measurements. In recent years, liquid–liquid interfaces have been investigated by spectroscopies such as total internal reflection spectroscopy (TIR), second harmonic generation (SHG), and sum frequency generation (SFG). Electrochemical methods and computer simulation techniques such as Monte Carlo and molecular dynamics have been used.

Acridine orange was choosen as a probe molecule, since heterocyclic compounds have low-lying n, π^* transitions. It is well known that rigid planar molecules have a high quantum yield of fluorescence; acridine orange is one of these molecules. It is used as a photosensitizer as well. Acridine orange is sensitive to fluorescence, has a low distribution to the organic phase, and has suitable criteria of luminescence such as the emission intensity at maximum wavelength, quantum yield, and lifetime. The lone pair at the nitrogen atom has an intimate relation to the fluorescence process. Acridine orange has C_{2v} symmetry axis through its cyclic nitrogen. To eliminate solvent interactions with the excited state of the solute molecule, n-heptane was choosen as the nonpolar phase (eliminating H-abstraction). The behavior of this orange dye was investigated at interfaces [1–3]. Studies of water–alkane interfaces were done using TIR and SHG [4]. The problem with TIR results is the high contribution from bulk. The best probability distribution function, *viz*, the chi-square of the obtained time-resolved spectrum, suggests at least two components of different lifetimes (see Fig. 1).

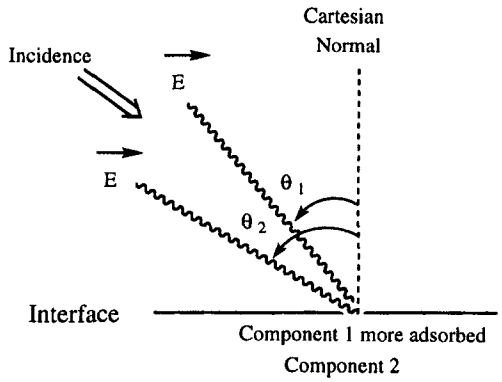

Fig. 1. In TIR measurements, when the incidence angle θ of the electric vector E from θ_1 to θ_2 is increased, the detected ratio of two adsorbed species (component 2/component 1) decreases. Possible components are excimer and exciplex.

Other obstacles are the low signal-to-noise ratios and the disadvantage of photoelectron time transit spread in time-correlated photon counting methods. The effect of different amphiphilic salts on two immiscible liquids, referred to as an interface between two immiscible electrolyte solutions (ITIES), was reported recently by Vincze *et al.* [5]. Stecki and Toxvaerd speculate that the interface is a sheet [6]. Burbage and Wirth propose the interface to be a rough plane [1], at which acridine orange can rotate either in the plane of the interface or out of the plane. Using the fluorescence emission to study the adsorption of acridine orange at the liquid–liquid interface, Burbage and Wirth [1] investigated the interface between water and some organic media. They found that there was a small range of orientation angles [3]. The variety of the species at the interface makes this judgment difficult, especially when the self-association process is possible, and we should consider the microenvironment. Acridine orange dye is positively charged, thus it is sensitive to a strong anion such as perchlorate; the dye cations make a nontransparent complex with anions in the solution.

This paper attempts to reconcile the available data. Acridine orange aggregates in aqueous solution, a fact proven using different spectroscopies [7–10]. A study of the aqueous association and the self quenching of 3,6-diaminoacridine (trivial name, proflavine) also was reported [11].

To form a stable dimer avoiding the repulsion of methyl groups, one monomer should tilt and give the dimer shape (Fig. 2). The thermodynamic dimerization constant K is equal to the activity of the dimer species divided by the square of the monomeric species. K in water is of the order of 10^{-4} dm^3 mol^{-1}. The two molecules which form the dimer are not parallel; instead, there is an inclined angle between them [8], which was reported to be 49°. In a recent study, the distance between the two molecules in the dimer was 14 Å [12]. In a study of surfactants at the interface [13], the negatively charged micelle surface traps cations. Polarity is related to aggregation.

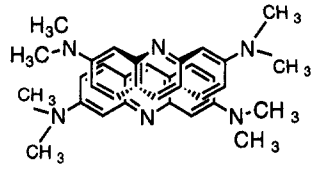

Fig. 2. The dimer formation of acridine orange; π–π overlap between the two molecules is preserved. The positive cyclic nitrogen on one molecule and the π negative electronic density on the other molecule stabilize the dimer. Steric hindrance is avoided as well.

This work shows that fluorescence emission is a useful tool to study adsorption at the liquid–liquid interface by analyses of both pH dependence and concentration dependence. The effect of the dodecyl aliphatic chain is proved to enhance the aggregation at the organic/aqueous interface

EXPERIMENTAL

Materials

Water and *n*-heptane were chosen as two solutions for the liquid–liquid interface. Acridine orange was obtained from Wako Pure Chemical Industries, Ltd., and was recrystallized from methyl alcohol [9]. The IR spectrum of the dye after purification was recorded using a KBr disk, streching and binding frequencies of the formula were distinguished. For measurments of fluorescence, a concentration of 1×10^{-7} *M* chromophore was found to minimize solute–solute interaction. *N*-Cyclohexyl-3-aminopropanesulfonic acid (CAPS) was obtained from Dojindo Laboratory. Heptane, spectroscopy grade, was obtained from Nacalai Tesque, purified through a column of fine silica to exclude polar fragments. Water used as an aqueous phase was Milli Q water whose resistance was 18.9 *M*Ω-cm.

Instruments

Absorption spectra were measured with a JASCO Model V-570 UV-VIS-NIR spectrophotometer. Steady-state fluorescence spectra were measured with a JASCO Model FP-770 using a quartz cuvette containing degassed solutions. Total internal reflection measurements were done in a cell as illustrated in Fig 3. Under the present experimental conditions, the liquid phase above the interface is *n*-heptane in which an excitation beam passes. It was confirmed that the heptane phase gave no detectable fluorescence, that is, no signal. Thus, the excitation spectra observed under TIR conditions can be ascribed to acridine orange adsorbed on the interface.

Interfacial tensions were measured using a programmable pulse motor driver (PPMD-805) with a stepping motor connected to a NEC personal computer (PC-9801). The resolution of time measurements was 10^{-2} s. All measurements were done at 25°C. A Rotavapor R-114 was used as a rotary evaporator for toluene, which was used as a solvent in the synthesis.

Synthesis of *N*-10-Dodecyl Acridine Orange

N-10-Dodecyl acridine orange was synthesized by the reaction of equimolar amounts of recrystallized acridine orange with n-$C_{10}H_{21}Br$. The π electronic density at the cyclic nitrogn atom in acridine is 1.17 [17]; this high value makes the reaction easy.

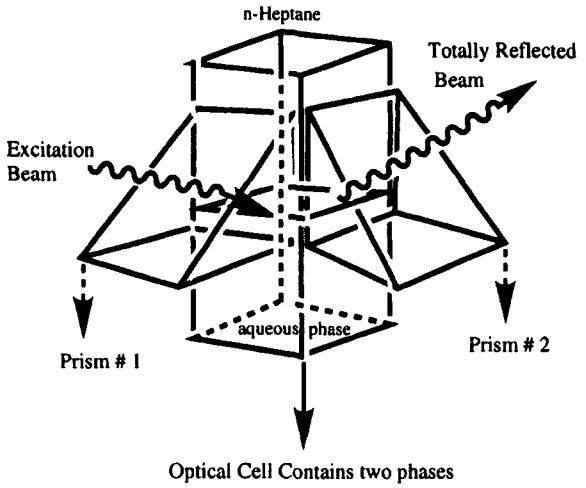

Fig. 3. Schematic drawing of the optical arrangement for measurements at the liquid–liquid interface.

Toluene was used as a solvent for synthesis, with reflux cooling the vapor while heating on a bath of paraffin oil. The reaction temperature was ca. 110°C. After several hours the product was confirmed by measuring the R_f value. Thin-layer chromatography was done on homogeneous, high-level-of-confidence sheets of alumina, then processed with a 366-nm UV lamp. Toluene was evaporated using a rotary evaporator over a warm water bath.

Interfacial Study

There are two major methods to measure the tension force at the interfacial region: the static drop volume method of Toruberg and the dynamic drop volume method of Jho and Burke [16]. The latter method depends on measuring the required time of a falling drop using a computer program. First, the linear relation between flow rate and step rate in the range 500–2000 lb s^{-1} was verified. The linear regression factor R was very close to unity.

The molar concentration of a solute per square unit of area Γ at the interface can be calculated from Eq. (1):

$$\Gamma = -\frac{1}{RT}\left(\frac{d\ln\gamma_e}{d\ln C}\right)_T \qquad (1)$$

where γ_e is the equilibrium value of the interfacial tension (γ_e is the intercept of the straight line with the ordinate axes; different concentrations give different γ_e values) for any concentration C at a fixed temperature T.

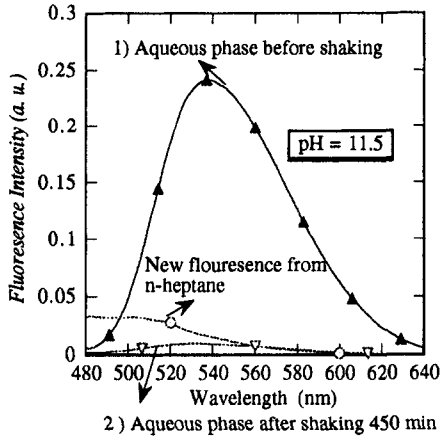

Fig. 4. Effect of shaking aqueous acridine orange (2×10^{-7} mol dm^{-3} which contains 1×10^{-2} mol dm^{-3} CAPS buffer) with *n*-heptane. pH adjusted to 11.5 with 1×10^{-1} mol dm^{-3} NaOH. Before shaking (▲) and after shaking for 450 min (▽). The fluorescence decrement of the aqueous phase shows that neutral species of acridine orange could migrate to the organic phase.

RESULTS AND DISCUSSION

In steady-state fluoresence for the bulk solution, the isoemissive point was found to be at 491 nm. To observe the ease of species motion across the interface, cation and neutral species were shaken separately. Figure 4 shows the fluoresence depletion of the aqueous phase, pH 11.5 (neutral species), after shaking with *n*-heptane (equal volumes of each): neutral species could cross the interface (new fluorescence from heptane). Figure 5 shows the fluorescence after shaking of cation species (pH 2): cation species are not able to cross the interface. Absorbance measurements showed data similar to those on fluoresence.

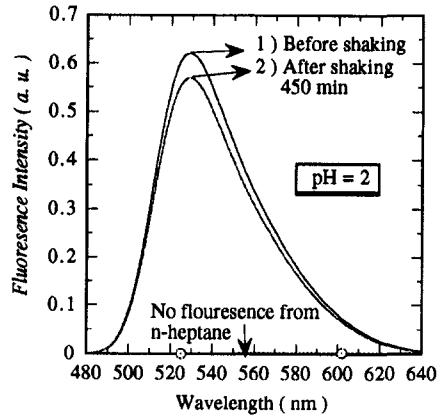

Fig. 5. Effect of shaking of 1×10^{-5} mol dm^{-3} acridine orange containing 1×10^{-2} mol dm^{-3} CAPS with *n*-heptane, pH 2. This figure shows that cationic species could not cross the interface.

Polarization is defined by Eq. (2)

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp}) \quad (2)$$

where $I_{\parallel}$ and $I_{\perp}$ are components of fluorescent light parallel and perpendicular, respectively, to the direction of polarization of the exciting light. Measurements were made at a right angle to the incident beam, through a high-pass filter, to eliminate the scattered excitation light.

Table I shows the results of measurement of polarization of an acridine orange bulk solution using a commercially available polarizing plate. In the fluorescence instrument, when the excitation ray passes through the polarizing plate, the solution in the cuvette will be excited by one component of the light [18].

Using a Ti:sapphire laser with a CCD camera, the time-resolved fluorescence decay curve of the purified acridine orange aqueous solution showed a steep decrease in counts of fluorescence trails after ca. 9 ns. This represents the decrease in luminescence intensity to the reciprocal of the naperian, e^{-1} of its value, i.e., to 37%. It is also the reciprocal of the radiative rate constant, which is the rate of decay of emission k_e^0 from the zero vibrational level of the first excited state S_1.

The magnitude of the fluorescence signal that comes from a dissolved dye in the evanescent wave away from the interface is related to the penetration depth (d_p) by Eq. (3):

$$d_p = [\lambda(n_1^2 \sin^2\theta - n_2^2) - 0.5]/2\pi \quad (3)$$

where λ is the incident wavelength, n_1 is the refractive index of the the upper phase, θ is the incidence angle, and n_2 is the refractive index of the lower phase.

At a pH value that is higher than the value of pK_a, species in the solution are neutral; neutral species have a relatively higher density of electrons than cations do. Neutral species require a higher energy for their absorbance; they absorb at a shorter wavelength. Neutral species of acridine orange absorb at 450 nm, the wide peak in absorbance spectra for dyes, the so called γ-peak [7]. However, Franck–Condon selection rules control the electronic transition. The pK_a in the ground unexcited state is less than its value in the triplet excited state, but

Table I. Mean of Three Fluorescence Polarization Values of Acridine Orange Using Steady-state Measurements When Inserting a Polarizing Plate Through the Excitation Light Before It Passed to the Sample, Then Using One Component of the Light

$I_{\parallel}$	$I_{\perp}$	$I_{\parallel} - I_{\perp}$	$I_{\parallel} + I_{\perp}$	P [from Eq. (2)]
20.98	9.149	11.831	30.129	0.393

the pK_a in the excited singlet state is the highest. Excitation makes the molecule much more basic and there is a redistribution of the charge between the ground and the excited states. During excitation an interaction between the unexcited singlet state and the first excited singlet state may result in two triplets. There are many excitation axes in an aromatic ring; the longest axis results in the maximum excitation.

Since the dimer fluorescence of acridine orange is less intense than that of the monomer, the dimer is of the "sandwich" type [7]. When the dimer fluorescence intensity is strong, e.g., as in isocyanine dyes, the dimer is of the "end-on" type [7]. Concentration has a role in stacking and partial dissociation. To form a dimer of two molecules, first, the two molecules should be capable of stacking, and second, they should have a low degree of hydrophobicity (in aqueous solutions the force of repulsion is 6.24×10^{-30} Cm). At a suitable pH value, intramolecular proton transfer is possible during the excited state.

The solvated solute molecule prefers to adsorb when the adsorption kinetic rate k_1 is high, and higher than the desorption rate k_2. The competition between adsorption and desorption at the site of adsorption will determine whether or not adsorption occurs. The diffusion coefficient (cm s^{-2}) at the interface is expected to be lower than the diffusion coefficient in the bulk because of the presence of *forces of association* at the interfacial layer. The interfacial layer is a mixture of both bulks. The interface boundary is a film of a finite thickness of nanometer order, and if it is thick enough, then carrier molecules can be located at the interface for a diffusion-controlled reaction. The carrier molecule works as a host to incubate a guest, then chelation is possible and mass transfer across the interface will be feasible. Stirring increases the interfacial area, allowing better mass transfer in industrial separation and liquid–liquid extraction processes. Part of the adsorbed molecule can penetrate into the other phase.

In analysis of excitation and emission, the excited-state constant is defined as the ratio of slope/intercept [15]. For acridine orange this constant was calculated and found to be 2.6×10^{-5} dm^3 mol^{-1}.

To analyze the effect of an aliphatic chain, Fig. 6 shows the absorbance spectra of a dodecyl derivative. In the case of dodecyl acridine orange, the dodecyl chain made extraction to *n*-heptane feasible. The heptane phase became colored after extraction.

For analysis of acid–base equilibria at the interface by fluorescence spectroscopy, excitation spectra (excitation spectra are uniqe to absorbance spectra) of acridine orange at the interface (using the cell in Fig. 3) were measured for three pH values. These three values were

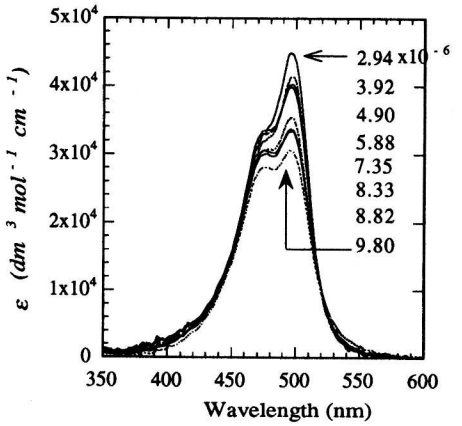

Fig. 6. Absorbance of $3–10 \times 10^{-6}$ mol dm^{-3} of 3,6-bis(dimethylamino)-10-dodecylacridinium bromide in 10^{-2} mol dm^{-3} HCl from aqueous bulk.

selected to be before and after the measured pK_a. The measured pK_a, where K_a is the acid-dissociation constant, was found to be 9.6. from the data on absorbance in bulk of neutral acridine orange and of cationic acridine orange. Three pH values were chosen: 7.68, 8.99, and 9.82. The three spectra of fluorescence excitation from the interface using the cell in Fig. 3 are shown in Fig. 7.

In Fig. 7 there are two peaks, one at 490 nm and the other at 415 nm. It is well known that in bulk solution, the absorbance peak at 490 nm is ascribed to the monomer species, and the absorbance peak at 470 is ascribed to the dimer species. At the interface, the band at 490 nm still exists and is ascribed to the cationic form of acridine orange; the peak at 490 nm was intense at pH 7.68, a value lower than 9.6. At 450 nm the intensity is highest for a pH value higher than 9.6 (9.82). The band at 415 nm is ascribed to the neutral form of acridine orange

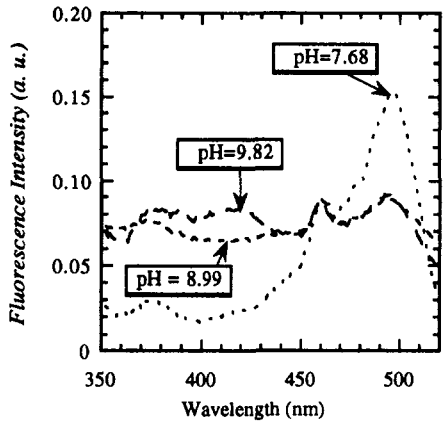

Fig. 7. pH effect on emission from the interfacial layer. The pH values are around the measured pK_a, at 7.68, 8.99, and 9.82.

because when the pH value is increased to 9.82, the band appeared at 415 nm. The fluorescent band shift to blue (from 470 nm in bulk, to the new band at the interface at 415 nm) means greater aggregation. For cationic species both in bulk and at the interface the band at 490 nm shows no shift in its wavelength. This result may show the difference in microenvironments between the cationic and the neutral forms of the adsorbed molecules the interface system. The clear-cut conclusion is that neutral species adsorb to the interface more than cationic species do. To my knowledge, this is the first approach to distinguishing among different species at the interface. This conclusion is in good agreement with the extraction result, that neutral species (pH 11.5) could pass through the interface (*vide supra* Fig. 4)

For interfacial tension, from Eq. (1), we first calculate the values of equilibrium tension γ_e for each concentration. The values of straight-line intercepts with the ordinate axis which are γ_e are calculated for several concentrations, as shown in Fig. 8.

Calculating $R = 8.3$ J mol^{-1} K^{-1} for the constant and 298 K for the ambient temperature, we derive Fig. 9 from Eq. (1).

The interfacial tension results mean that the concentration at the interface increases with increases in the concentration of bulk water, till reaching a saturation value when the bulk concentration is high enough: 10–12 $\times$ 10^{-4} mol dm^{-3}.

The ionic strength (I) is related to the concentration (C) for the charge (Z) for all species (i) by the following equation:

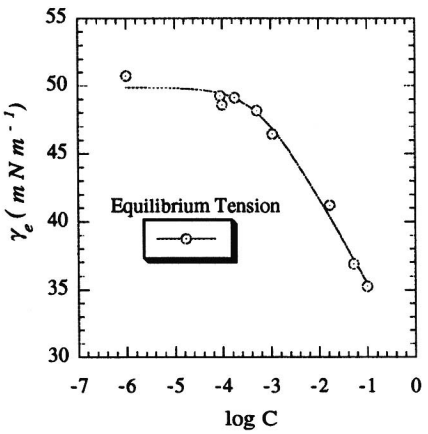

Fig. 8. Gibbs plot of the equilibrium liquid/liquid interfacial tension for different concentrations of acridine orange in water, ranging from 1 $\times$ 10^{-6} to 1 $\times$ 10^{-1} mol dm^{-3}. The organic phase is *n*-heptane. Values of the equilibrium interfacial tension γ_e for each concentration were used.

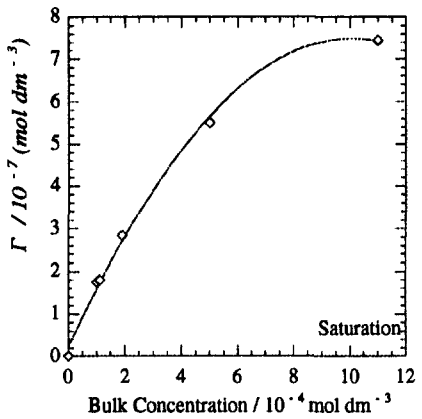

Fig. 9. Interfacial concentration of adsorbed acridine orange at the water/*n*-heptane interface versus acridine orange concentration in bulk. Saturation at the interface is at a high concentration of solute.

$$I = (\Sigma C_i Z_i^2/2) \tag{4}$$

The diffusion coefficient in bulk water, D_b, is expected to be much higher than that at the interface, D_i (cm^2/s^{-1}), since there are hindering forces at the interface. The D value change order is onefold. On the other hand, the higher viscosity η will lower the D value directly since the encounter rate constant of the diffusion process is

$$k_D = 8RT/3000\ \eta \tag{5}$$

The distance of diffusion in liquids (μm) during the lifetime of the excited species, τ, i.e., during a few nanoseconds, is related to the value of the square root of $2D\tau$. Fick's laws of diffusion are also applicable. However, the Gouy–Chapman theory for the ionic distribution model is more specific for interfaces [19]. In concentrated solutions of a solute which has long aliphatic chains, there is association which may be disrupted by mechanical shear when excited, then reestablished within a short time.

ACKNOWLEDGMENTS

I would like to thank all persons in charge who made this contribution possible. Special thanks go to the Ministry of Education, Sport, and Culture in Japan.

REFERENCES

1. (a) J. D. Burbage and M. J. Wirth (1992) *J. Phys. Chem.* **96**, 5943–5948. (b) J. D. Burbage and M. J. Wirth (1992) *J. Phys. Chem.* **96**, 9022–9025.

2. D. A. Piasecki and M. J. Wirth (1993) *J. Phys. Chem.* **97**, 7700–7705.
3. J. M. Kovaleski and M. J. Wirth (1995) *J. Phys. Chem.* **99**, 4091–4095.
4. J. C. Conboy, J. L. Daschbach, and G. L. Richmond (1994) *J. Phys. Chem.* **98**, 9688–9692.
5. A. Vincze, G. Horavai, and F. A. M. Leermakers (1995) *Electrochim. Acta* **40**, 2875–2879.
6. J. Stecki and S. Toxvaerd (1995) *J. Chem. Phys.* **103**, 4352–4359.
7. B. C. Burdet (1983) in E. Wyn-Jones and J. Gormally (Eds.), *Studies in Physical and Theoritical Chemistry, Aggregation Processes in Solution*, Vol. **26**, Elsevier, Amsterdam, pp. 260, 276, 278.
8. D. B. Davies, L. N. Djimant, and A. N. Veselkov (1996) *J. Chem. Soc. Faraday Trans.* **92**, 383–390, and references therein.
9. B. H. Robinson, A. Löffler, and G. Schwarz (1973) *J. Chem. Soc. Faraday Trans. 1* **69**, 56–69.
10. B. H. Robinson, A. Seelig-Löffler, and G. Schwarz (1975) *J. Chem. Soc. Faraday Trans. 1* **71**, 815–830.
11. G. R. Haugen and W. H. Melhuish (1964) *Trans. Faraday Soc.* **60**, 386–394.
12. X. He, X. Feng, G. Zhange, and H. Shi (1995) *Fenxi Huaxue*, i.e., *Chin. J. Anal. Chem.* **22**(6), 565–568 (in Chinese with an English abstract).
13. M. Wong, J. K. Thomas, and T. Nowak (1977) *J. Am. Chem. Soc.* **99**, 4730–4735.
14. M. Hamed, E. Abdalla, and M. Mahmoud (1994) *Can. J. Appl. Spectrosc.* **39**, 126–131.
15. G. D. Correll, R. N. Cheser III, F. Nome, and J. H. Fendler (1978) *J. Am. Chem. Soc.* **100**, 1254–1262.
16. Z. Samec, V. Marecek, and D. Homolka (1981) *J. Electroanal. Chem. Interfac. Electrochem.* **126**, 121–129.
17. (a) R. Acheson (1973) *Acridines*, 2nd ed., Interscience, New York. (b) E. Rood (1957) *Chemistry of Carbon Compounds, IV(A)*, Elsevier, Amsterdam, p. 671.
18. J. Schreiber and M. P. Daune (1974) *J. Mol. Biol.* **83**, 487–501.
19. For more details on interfaces see (a) B. G. Cox (1994) *Modern Liquid Phase Kinetics*, Zeneca, Oxford; (b) C. Gutierrez and C. Melendres (1990) *Spectroscopic and Diffraction Techniques in Interfacial Electrochemistry*, NATO Advanced Science Institute Series C, Vol. **320**, Kluwer Academic, Dordrecht; (c) B. Derjaguin (1987) in J. Kitchener (Ed.), *Surface Forces*, Plenum, New York.