

TRANSIENT EFFECTS IN QUENCHING DETECTED BY HARMONIC-CONTENT FREQUENCY-DOMAIN FLUOROMETRY

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Transient effects in the diffusional quenching of indole fluorescence by acrylamide were detected by frequency-domain fluorometry. The samples are excited with a repetitive train of picosecond pulses, and the frequency response is measured using the harmonic content up to 1 GHz. The decay of unquenched indole in aqueous buffer is dominantly a single exponential. In the presence of acrylamide the partially quenched indole fluorescence became distinctly more complex. Analysis of the frequency response of the fluorescence yields the apparent interaction radius (7.1 Å) and mutual diffusion coefficient (1.1×10^{-5} cm²/s).

1. Introduction

Dynamic or collisional quenching of fluorescence is frequently used to study diffusion in homogeneous solution and in more complex systems involving biological macromolecules. The degree of quenching depends upon the rate of collisional encounters of the fluorophore with the quencher. Consequently, quenching is dependent upon the rate of quencher and fluorophore diffusion and the accessibility of the fluorophore to contact with the quencher. In biochemistry, fluorescence quenching has been used to estimate the diffusion rates and local concentration of quenchers in proteins and membranes [1–5].

Fluorescence quenching data are often evaluated in terms of the diffusion-controlled rate constants for quenching, which are assumed to be time independent. However, the quenching constant is expected to be time dependent at short times [6–8]. These transient effects are expected to result in non-exponential decays of fluorescence, which are often undetected due to the limited time resolution of the time-resolved

data or due to the sole use of steady state measurements. Transient effects are thought to be most apparent for relatively slow diffusion in viscous solvents and for longer fluorescence decay times. In fact, the only measurement of a quenching-induced non-exponential decay was for such a system, with a decay time of 40 ns and a diffusion coefficient of 0.03×10^{-5} cm²/s [9,10]. Within the past several years, we and others introduced the technique of frequency-domain fluorometry [11,12]. The impulse response or intensity decay of the sample is recovered from the frequency response, typically measured from 1 to 200 MHz. More recently, we increased the ability to recover complex and rapid (subnanosecond) decays of fluorescence by increasing the frequency range to 2 GHz. This was accomplished by using a faster detector, a microchannel plate photomultiplier, and the intrinsic harmonic content of the psec pulse train from a laser source [13]. The data from this new instrument allowed us to detect the transient effects in non-viscous solutions with decay times near 2 ns. The data at low concentrations of quencher yielded non-exponential decays which agreed with the Smoluchowski model. The high apparent resolution suggests that still more complex models for diffusion can be tested by gigahertz frequency-domain fluorometry.

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2. Theory

Assume the fluorescence in the absence of externally added quencher decays as a single exponential

$$I(t) = I_0 \exp(-t/\tau_0), \quad (1)$$

where τ_0 is the unquenched decay time. Collisional quenching is an additional rate process which depopulates the excited state. Generally, this rate constant (k) is assumed to be independent of time. The additional depopulation rate constant is

$$k = 4\pi RN'D, \quad (2)$$

where R is the interaction radii (sum of the radius of the fluorophore (F) and quencher (Q)), $N' = 6.02 \times 10^{20}$, and D is the sum of the F and Q diffusion coefficients. For a quenching constant which is independent of time, the decay in the presence of quencher is predicted to remain a single exponential with a lifetime (τ)

$$1/\tau = 1/\tau_0 + k[Q], \quad (3)$$

where $[Q]$ is the molar quencher concentration. However, eq. (2) is only approximately correct, and k is expected to be itself dependent on time [6–8]. The time dependence originates with the random distribution of fluorophores and quenchers. The fluorophores with a closely located quencher are extinguished rapidly. With time following excitation, the ensemble of fluorophores evolves, and the fluorophores which remain in the excited state are those which are distant from the closest quencher. Hence, the time-dependent rate constant decreases from an initially high value to the diffusion limited value (eq. (2)). An approximate expression for $k(t)$ is

$$k(t) = 4\pi RN'D [1 + R(\pi Dt)^{-1/2}]. \quad (4)$$

The time-dependent quenching rate results in a non-exponential decay [9,10]. This decay can be obtained by integration of the differential equation describing $dI(t)/dt$ which includes the time-dependent rate constant. This yields

$$I(t) = I_0 \exp(-t/\tau - bt^{1/2}), \quad (5)$$

where τ is given by eq. (3) and

$$b = 4R^2N'(\pi D)^{1/2}[Q]. \quad (6)$$

We compared the frequency response of quenched in-

dole solutions with the response predicted from eqs. (4)–(6).

The frequency response is characterized by the frequency- (ω) dependent values of the phase shift (ϕ_ω) and the extent of demodulation (m_ω). The parameters describing the decay law are estimated by comparison of predicted (c) values of ϕ_ω ($\phi_{c\omega}$) and m_ω ($m_{c\omega}$) with the measured values. For any decay law the predicted phase and modulation values are given by

$$\phi_{c\omega} = \arctan(N_{c\omega}/D_{c\omega}), \quad (7)$$

$$m_{c\omega} = (N_{c\omega}^2 + D_{c\omega}^2)^{1/2}, \quad (8)$$

where

$$N_{c\omega} = \int_0^\infty I(t) \sin \omega t dt / \int_0^\infty I(t) dt, \quad (9)$$

$$D_{c\omega} = \int_0^\infty I(t) \cos \omega t dt / \int_0^\infty I(t) dt. \quad (10)$$

The goodness-of-fit is characterized by

$$\chi_R^2 = \frac{1}{\nu} \sum_\omega [(\phi_\omega - \phi_{c\omega})/\sigma_\phi]^2 + \frac{1}{\nu} \sum_\omega [(m_\omega - m_{c\omega})/\sigma_m]^2, \quad (11)$$

where ν is the number of degrees of freedom.

3. Materials and methods

The frequency-domain data were analyzed by non-linear least squares, as described by Johnson and Frasier [14]. Our algorithm uses an adaptive Newton–Cotes nine-point quadrature numerical integration routine [15] for the decay law with the $t^{1/2}$ term (eqs. (9) and (10)), and analytical expressions for the single exponential decay. In any least-squares analysis the parameters may be correlated, that is, the value of χ_R^2 may remain nearly constant for compensating changes in the parameter values. Our algorithm estimates the uncertainties in the recovered parameters (R and D) by a method which accounts for correlation between the parameters [14,16].

Frequency-domain data were obtained using the instrument described previously [11–13]. Indole was

excited at 295 nm using a 3.7931 MHz train of 5 ps pulses from a frequency-doubled and cavity-dumped dye laser with rhodamine 6G as the dye. The pump was a mode-locked, argon ion laser, 514 nm. Modulated emission can be measured at any integer multiple of 3.7931 MHz. Measurements to 2 GHz are possible with a microchannel plate photomultiplier tube (Hamamatsu R-1564 U). Indole emission was observed through a Schott WG320 filter. All measurements were at 20°C in 0.05 M tris buffer, $pH = 7.5$. Polarizers at the magic angle orientation were used to eliminate the effects of Brownian rotation on the intensity decays. The value of χ_R^2 was calculated using estimated uncertainties of 0.2° for phase and 0.005 for modulation. These values were found appropriate from about two years of experimentation with a variety of experimental configurations [11,13,17–19].

4. Results

The frequency response of indole in the absence of the quencher acrylamide is shown in fig. 1. The phase angles increase and the modulation values decrease with increasing modulation frequency. The upper frequency limit is determined primarily by the "bandwidth" of the sample. That is, the degree of demodulation due to the relatively long decay time of indole limits the measurements to 159 MHz. The solid line shows the best single exponential fit to the data, and the lower panels show the deviations. The small and random deviations and the modest value of $\chi_R^2 = 2.2$ (table 1) indicates the decay of indole is close to a

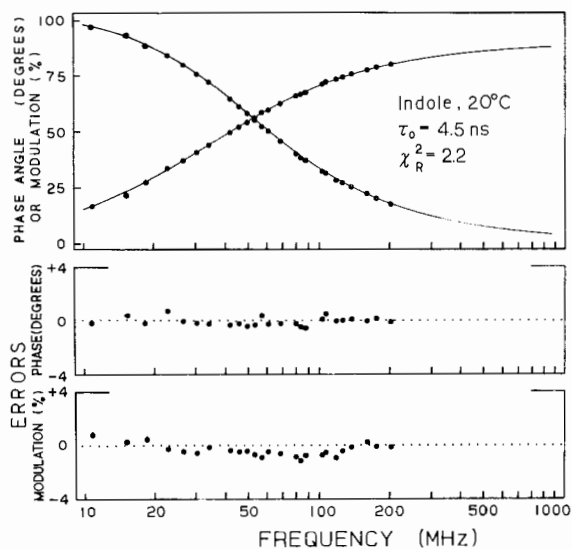


Fig. 1. Frequency response of indole in the absence of quenching. The symbols (●) indicate the data, and the solid line represents the best single decay time fit. The lower panels show the deviations between the data and the best fit.

single exponential. Double exponential fits yield only minor decreases in χ_R^2 , and the second decay time is either close to 4.5 ns or of small amplitude. For the present analysis the indole decay is adequately described as a single exponential. We note that our values of χ_R^2 are not necessarily near unity because the errors (σ_ϕ and σ_m , eq. (11)) are only approximately known. Nonetheless, the relative values of χ_R^2 can be compared, independent of the small uncertainty in σ_ϕ and σ_m .

The frequency response of indole in the presence

Table 1
Acrylamide quenching of indole

[acrylamide] (mM)	$\exp(-t/\tau)$		R (Å)	$\exp(-t/\tau - 2bt^{1/2})$	
	τ (ns)	χ_R^2 ^{a)}		$10^5 D$ (cm ² /s)	χ_R^2
0	4.50	2.2	—	—	—
25	2.42	7.3	6.5 (5.6–7.4) ^{b)}	1.24 (1.04–1.48)	1.8
50	1.62	19.7	7.2 (6.6–7.8)	1.06 (0.94–1.20)	1.7
75	1.19	42.4	7.6 (7.1–8.1)	0.94 (0.87–1.06)	1.9
0–75	—	—	7.1 (6.7–7.6)	1.07 (0.98–1.18)	2.0

a) Calculated using $\sigma_\phi = 0.2^\circ$ and $\sigma_m = 0.005$.

b) The values in parentheses are the range of values (uncertainties) consistent with the data, including effects of correlation between the parameters [14–16].

of 0.075 M acrylamide shows deviations from a single exponential decay (fig. 2). This is seen from the inadequacy of the single exponential model (—), the systematic deviations from this model (\circ), and the twentyfold elevation in χ_R^2 to 42.4. It should be noted that the degree of quenching is rather modest (about fourfold), and the solution is non-viscous. Because of the shorter mean decay time in the presence of quenching the frequency response was measurable to 811 MHz. Similar but less dramatic deviations from a single exponential decay were seen at lower quencher concentrations (table 1). The value of χ_R^2 for the single exponential fit increases monotonically with the acrylamide concentration. These results indicated that the decays of indole became more complex in the presence of quenching. Similar increases in complexity were seen for indole quenched by iodide and for 1,2-benzanthracene quenched by carbon tetrabromide (results not shown). Evidently, the frequency-domain method can readily detect transient quenching effects, even for the difficult case of indole in aqueous solution, and for degrees of quenching near twofold.

The data were then analyzed with eq. (5), which is

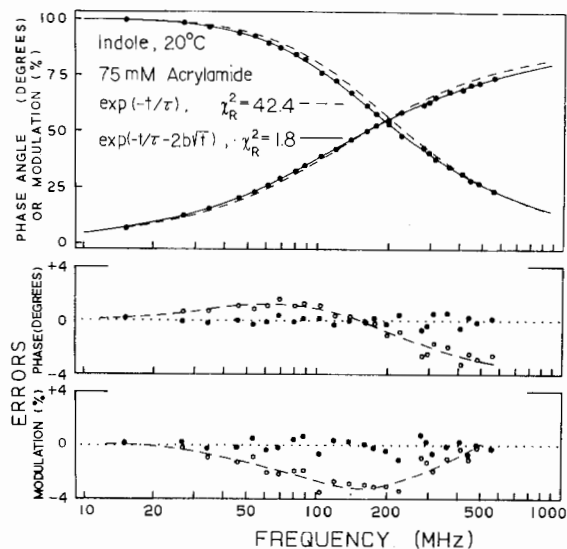


Fig. 2. Frequency response of indole in the presence of 0.075 M acrylamide. The solid line indicates the best fit obtained using $\exp(-t/\tau - 2bt^{1/2})$. The dashed line indicates the best fit using a single decay time ($\exp(-t/\tau)$). The lower panels show the deviations for the best single exponential fit (\circ) and for $\exp(-t/\tau - 2bt^{1/2})$ (\bullet).

the approximate form of the decay expected at low degrees of quenching. This model is consistent with the data, as seen by the good match to the data, the small deviations (fig. 2, \bullet), and the small values of χ_R^2 (table 1). These values of χ_R^2 near 2 are the lowest obtainable even for double or triple exponential fits, so there is no justification to accept a decay law with more parameters than eq. (5). It should be noted that the same values of R and D were recovered at each acrylamide concentration (table 1).

We then questioned whether all four sets of data, at acrylamide concentrations of 0, 25, 50 and 75 mM, were consistent with the same decay law. Such consistency cannot be obtained with a single sum of exponentials because the decay times are different at each concentration of quencher. For example, twelve parameters (eight decay times and four independent amplitudes) would be required to fit each of the four data sets to a bi-exponential decay. However, the data should all be consistent with eq. (5) because the fitting parameters, R and D , are expected to be the same. The concentration of quencher should account for the differences in the frequency response at each acrylamide concentration. Simultaneous analysis of all the data indicated they were consistent with eq. (5). This is evident from the good match of all the data to the four theoretical curves (fig. 3), the consistent values

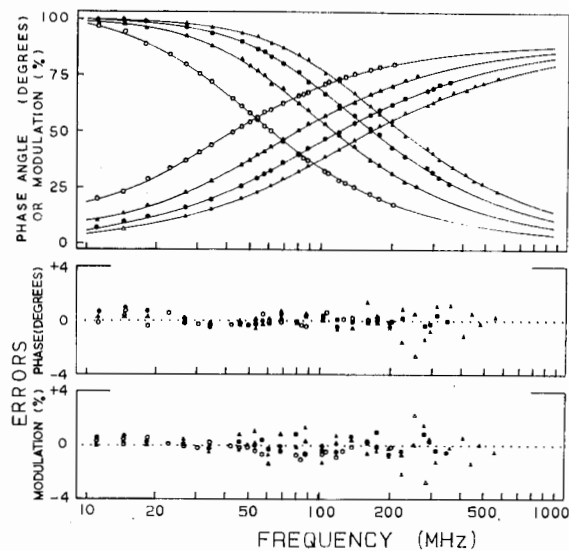


Fig. 3. Simultaneous fit of quenched indole with acrylamide concentrations of 0 (\circ), 25 (\blacktriangle), 50 (\bullet) and 75 mM (\triangle). The lower panel shows the deviations from the simultaneous fit.

of R and D , and the acceptable value of χ_R^2 . We conclude that these experimental data are consistent with the decay law expected from a time-dependent quenching constant (eq. (5)).

For accuracy we note that eq. (4), and as a consequence eq. (5), is only an approximation and that quenching is expected to result in still more complex intensity decays. In fact, we observed deviations from eq. (5) at quencher concentrations above 0.1 M, for both iodide and acrylamide quenching of indole [20].

The value of the encounter radius (R) and the diffusion coefficient (D) which we discovered (table 1) are consistent with the expected values. Based upon the molecular structures, Eftink and Ghiron calculated an encounter radius of 6 to 7 Å for indole and acrylamide [21]. A diffusion coefficient near 1×10^{-5} cm²/s is closely comparable with that of similarly sized molecules such as acetamide in water at 20°C, 1×10^{-5} cm²/s [22,23] or propional in water at 20°C, 0.9×10^{-5} cm²/s [24].

5. Discussion

The results demonstrate that the transient effects in fluorescence quenching can be easily detected from the frequency response of the emission. We expect this capability to be valuable in studies of quencher diffusion in homogeneous solution and through macromolecules. For instance, deviations of the frequency response from that predicted by $\exp(-t/\tau - 2bt^{1/2})$ may yield additional details about diffusive processes in solution, especially those occurring on the ps timescale. Prediction of the effects of diffusion on quenching has been the subject of theoretical studies for both solutions [24,25] and for macromolecules [26,27]. Cukier [25] predicted that the intensity decays remain a single exponential in the presence of quenching. This prediction does not agree with our experimental observation of more complex decays due to quenching. Hence, Cukier's theory is only correct at very low degrees of quenching, which are not experimentally useful, or the theory contains invalid assumptions or inappropriate approximations. Additionally, we found that the intensity decays became still more complex at higher concentrations of quencher. In fact, deviations from eq. (5) have already been observed with

higher degrees of quenching [20], so the data apparently contain additional information on diffusion and proximity effects in quenching. It seems probable that quenching of a fluorophore on the surface or within a macromolecule will also be distinct from eq. (5) due to a limited angular range of accessibility or due to barriers to diffusion at the macromolecule-water interface. We expect these phenomena to be measurable from the frequency responses of the emission.

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