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FREQUENCY-DOMAIN PHASE-MODULATION FLUOROMETRY: RESOLUTION OF COMPLEX DECAYS OF FLUORESCENCE INTENSITY AND ANISOTROPY

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We used frequency-domain fluorometry to resolve multi-exponential decays of fluorescence intensity and anisotropy. The samples are excited with intensity modulated light, at modulation frequencies ranging from 1 to 200 MHz, and we measure the phase-shifts and demodulation factors. The decay times of a three-component mixture were resolved, 1.2, 4.1 and 7.7 nsec, as well as the two anisotropy decay times of the anisotropic rotator perylene.

1. INTRODUCTION

Fluorescence spectroscopy is widely used in biochemical research because of its high sensitivity and the timescale of the phenomenon. Because of the complexity of biological macromolecules it is often necessary to resolve multi-exponential decays of fluorescence intensity or anisotropy. Time-resolved decays of intensity or anisotropy can, in principle, be determined using measurements in the time-domain or in the frequency-domain. In the time-domain one measures the time-dependent emission of the sample following pulsed excitation. In the frequency-domain one measures the phase angle and modulation of the emission relative to the intensity-modulated incident light.

Phase-modulation fluorometers were constructed prior to the development of time-domain instruments. It is generally recognized that these instruments are useful for measurement of subnanosecond lifetimes. However, most phase-modulation fluorometers operate at only one to three fixed modulation frequencies. Even with very precise data, the limited amount of data does not allow the resolution of complex decays of intensity or anisotropy. Frequency-domain phase-modulation fluorometers were recently constructed (1,2). These instruments allow measurement of the fluorescence phase angles (ϕ_ω) and demodulation factors (m_ω) at frequencies ranging to 200 MHz. ω is the circular modulation frequency. These data can be analyzed by the method of non-linear least squares to determine the intensity or anisotropy decay times of the samples (3,4).

2. THEORY

For a mixture of n fluorophores, the impulse function (I(t)) is a sum of exponential decays

$$I(t) = \sum_i^n \alpha_i e^{-t/\tau_i}. \qquad (1)$$

τ_i is the decay time of the i'th component and α_i is the preexponential factor. At each modulation frequency the expected values of ϕ_ω (= $\phi_{C\omega}$) and m_ω (= $m_{C\omega}$) are given by

$$\phi_{C\omega} = \arctan (N_\omega / D_\omega); \quad m_{C\omega} = (N_\omega^2 + D_\omega^2)^{1/2} \tag{2}$$

where

$$N_\omega \cdot J = \sum_i \frac{\alpha_i \omega \tau_i^2}{1 + \omega^2 \tau_i^2}; \quad D_\omega \cdot J = \sum_i \frac{\alpha_i \tau_i}{1 + \omega^2 \tau_i^2} \tag{3}$$

and $J = \sum_i \alpha_i \tau_i$. The values of α_i and τ_i are selected by minimization of χ^2,

$$\chi^2 = \sum_\omega \frac{1}{\sigma_{\phi\omega}^2} (\phi_\omega - \phi_{C\omega})^2 + \sum_\omega \frac{1}{\sigma_{m\omega}^2} (m_\omega - m_{C\omega})^2 \tag{4}$$

where $\sigma_{\phi\omega}$ and $\sigma_{m\omega}$ are the uncertainties of the measured phase and modulation values.

Time-resolved anisotropies can be determined from the phase angle difference (Δ_ω) between the perpendicular ($\phi_\perp$) and parallel ($\phi_\parallel$) components of the modulated emission ($\Delta_\omega = \phi_\perp - \phi_\parallel$) and the amplitude ratio (Λ_ω) of the parallel ($m_\parallel$) and the perpendicular ($m_\perp$) components of the modulated emission ($\Lambda = m_\parallel / m_\perp$). The expected values of Δ_ω ($\Delta_{C\omega}$) and Λ_ω ($\Lambda_{C\omega}$) can be calculated from the sine and cosine transforms of the individual polarized decays (5,6)

$$N_i = \int_0^\infty I_i(t) \sin \omega t \, dt; \quad D_i = \int_0^\infty I_i(t) \cos \omega t \, dt \tag{5}$$

The frequency-dependent values of Δ_ω and Λ_ω are given by

$$\Delta_{C\omega} = \arctan \left(\frac{D_\parallel N_\perp - N_\parallel D_\perp}{N_\parallel N_\perp + D_\parallel D_\perp} \right); \quad \Lambda_{C\omega} = \left(\frac{N_\parallel^2 + D_\parallel^2}{N_\perp^2 + D_\perp^2} \right)^{1/2} \tag{6}$$

The parameters describing the anisotropy decay are determined by minimizing χ^2.

3. RESULTS

To illustrate the resolution obtainable from the frequency-domain measurements we examined a two component mixture of anthracene (4.1 nsec) and 9,10-diphenylanthracene (6.3 nsec). For the forced single component fit (Figure 1, -o-) the deviation between the measured and calculated values vary systematically with frequency, and reduced χ^2 (χ_R^2) is too large, indicating the decay law is inappropriate. Inclusion of a second component in the decay law results in a 45-fold reduction in χ_R^2 and random deviations between the measured and calculated values (-●-). The lifetimes of the components agree with those measured for the pure solutions.

A more difficult resolution of a three-component mixture of POPOP,

anthracene and 9-vinylanthracene is shown on the right side of Figure 1. In this case incrementation of the decay law to include three components results in a ten-fold decrease in χ_R^2. Importantly, the decay times of 1.24, 4.13 and 7.70 nsec are in excellent agreement with the expected values. The resolution of such a mixture must be regarded as a difficult task.

It is frequently desirable to resolve multi-exponential decays of fluorescence anisotropy. Such complex decays result from segmental motions of protein-bound fluorophores, from the asymmetrical shapes of proteins and fluorophores, and from the anisotropic environments of lipid bilayers. Differential phase and modulation ratio data for perylene are shown in Figure 2. It is evident that these data cannot be fit using a single correlation time (--o--). Inclusion of two correlation times in the fitting algorithm results in a 8-fold decrease in χ_R^2 and more random deviations (--●--). The correlation times we observed were 1.5 and 10.8 nsec, with $r_0 g_1$ = 0.169 and $r_0 g_2$ = 0.184. These results are in rather good agreement with the time-resolved measurements; these values are 1.7 and 13 nsec, with $r_0 g_1$ = 0.1 and $r_0 g_2$ = 0.24 (7).

Frequency domain measurements were also used to determine the short correlation times of POPOP in fluid solvents (Figure 2). In this instance we used only the phase data. The solid lines represent the calculated values of Δ_ω for the correlation times shown on Figure 2. We could determine correlation times as short as 0.11 and 0.047 nsec for POPOP in ethanol and hexane at 45°C, respectively.

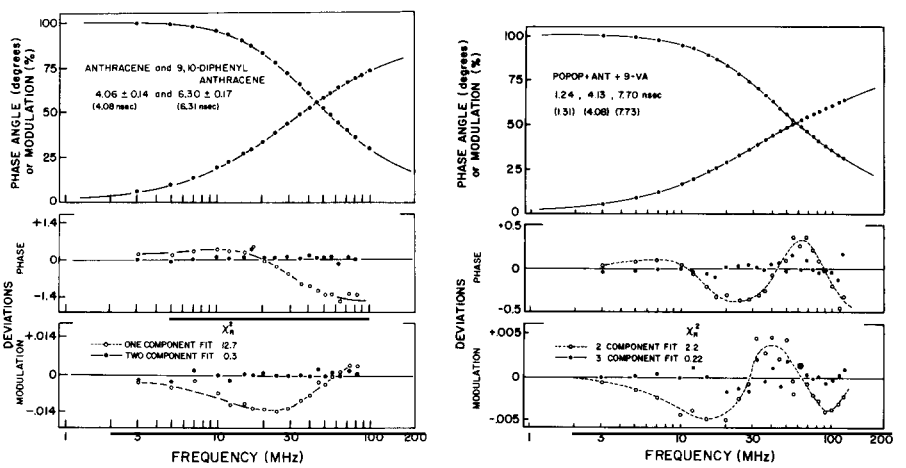

Figure 1. Left; Resolution of a Two-Component Mixture: Right; Resolution of a Three-Component Mixture. The expected values are given in brackets.

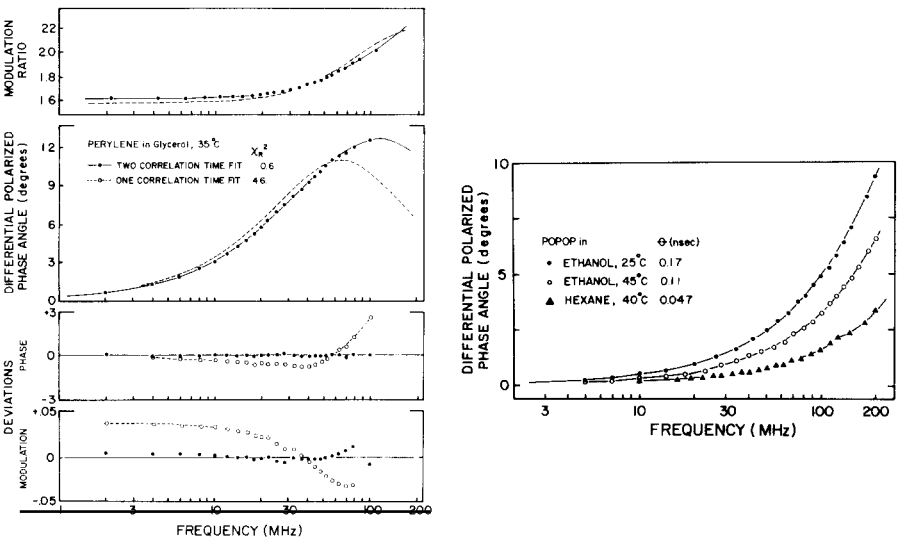

Figure 2. Left; Anisotropy Decay of Perylene: Right; Anisotropy Decay of POPOP in Fluid Solvents.

4. CONCLUSIONS

Frequency-domain fluorometry provided reliable resolution of complex fluorescence decays. The instrumentation is simple and seemingly free of systematic errors. We are hopeful that this technique we become widely utilized in chemical and biochemical research.

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