

Direct Determination of Kinetic Parameters for Diffusion-Influenced Reactions in Solution by Analysis of Fluorescence Decay Curves

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Fluorescence decay curves were synthesized using the Smoluchowski–Collins–Kimball (SCK) model for diffusion-influenced bimolecular reactions so as to simulate measurements using the technique of time-correlated single photon counting (TCSPC). The experimental conditions required for successful recovery of the parameters used to generate these decay curves were assessed by direct analysis using the decay function associated with the SCK model, where the term “direct analysis” refers to analysis in which all of the fundamental parameters associated with the SCK model are permitted to vary freely. Analysis of decay curves synthesized using an instrument response function measured using a flash-lamp TCSPC system and having a full-width at half-maximum (fwhm) of approximately 2.8 ns was unsuccessful for the values of the parameters associated with the SCK model that were used in this work. However, analysis of these synthesized fluorescence decay curves using the long-time approximation to the SCK model was found to be valid. The results of analysis of fluorescence decay curves synthesized using a 37-ps fwhm instrument response function indicate that the long-time approximation becomes a poor description of the kinetics of diffusion on the time scale associated with these decay curves. Furthermore, direct analysis using the function associated with the SCK model of the synthesized fluorescence decay curves convoluted with this response function and containing 5.0×10^4 or 1.0×10^5 counts in the channel of maximum intensity resulted in the recovery of parameter values that are in very good agreement with those used to generate these decay curves. The results obtained using the simple methodology developed in this work demonstrate for the first time that direct analysis of fluorescence decay curves measured using TCSPC according to the decay function derived from the SCK model can yield reliable estimates for values of the relevant parameters under suitable experimental conditions.

KEY WORDS: Decay curve analysis; diffusion-influenced reactions; Smoluchowski–Collins–Kimball model; simulated fluorescence decay curve; fluorescence quenching.

INTRODUCTION

The verification by experiment of equations describing the time dependence of the rate coefficient for

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diffusion-influenced bimolecular chemical reactions in solution $k(t)$ has been the subject of a number of recent publications [1–25]. The measurement of the decay of fluorescence from a fluorophore in the presence of a reactant that can quench the emitted fluorescence provides a powerful tool for the examination of the influence of diffusion on the kinetics of rapid reactions occurring in fluid solutions because it enables these reactions to be

probed on time scales over which diffusional effects will be most prominent. The decay of fluorescence from fluorophore solutions containing a known concentration of quencher has been measured using techniques such as time-correlated single photon counting (TCSPC) [1–17], fluorescence upconversion [8,11], and frequency-domain fluorometry [18,19], and has been analyzed according to the equations for $k(t)$ that are based on the Smoluchowski model [26,27], or the Smoluchowski model with the Collins–Kimball boundary condition (SCK model) [27,28]. The latter model is generally regarded as being more physically realistic because, unlike the Smoluchowski model, the value for the intrinsic reaction rate constant k_{act} is not assumed to be infinite. Analysis using other more sophisticated models has also been attempted recently [11,17].

The nonlinear least-squares analysis using the equation for $k(t)$ derived from the SCK model of data obtained by using these experimental techniques is not straightforward, due to correlations between the fitted parameters [7,10,13,18,19], and it has been found necessary to fix at least one of the fitted parameters in order to recover physically realistic values for the other parameters. Several methods have been applied to the analysis of fluorescence decay data obtained using the technique of TCSPC.

In one method [6,7], the time-zero shift parameter δ was used as a fixed parameter. The results of an extensive analysis using this method for the analysis of fluorescence decay data synthesized for TCSPC experiments have been reported by Das and Periasamy [7]. This approach was subsequently applied to the analysis of measured fluorescence decay curves [6]. The first step in this method of analysis was to demonstrate that a unique set of values for the fitted parameters, comprising values that were in good agreement with those values used to generate the original synthesized decay data, could be obtained by varying the value at which δ was fixed and then analyzing the synthesized decay curve at each fixed value of δ . The relative uncertainties in this set of parameter values were also calculated. Measured fluorescence decay curves were then analyzed using the same methodology. This resulted in sets of optimized values for the fitted parameters at each fixed value of δ . The magnitudes of the relative uncertainties in the values recovered from the analysis of the measured decay curves at each value of δ were then compared with the corresponding uncertainties in the unique set of results that was obtained from the analysis of the synthesized fluorescence decay curve as mentioned above. The set of values representing the best estimates of the true parameter values was then deemed to be that set which

contained relative uncertainties in the parameter values that were comparable with those for the set of parameter values recovered from the analysis of a range of synthesized decay curves. Although this approach was effective in recovering values for the optimized fitted parameters that were consistent with those values used for the calculation of synthesized fluorescence decay curves, as well as physically realistic values for the fitted parameters from the analysis of measured fluorescence decay curves, the methodology developed by these authors is inhibitive convoluted. The uncertainties in the recovered parameters were estimated from the diagonal elements of the error matrix. Uncertainties obtained in this way are usually regarded as underestimates of the true values [18,32]. This approach was unable to recover unambiguous values for the three fundamental parameters associated with the SCK model, due to the large uncertainties in the values recovered for the fitted parameters.

Another method, used later by the same authors [10], involves the fixing of a parameter which is a function of all three of the fundamental parameters of the SCK model instead of δ . The approach adopted in this method was to locate values for the remaining two fitted parameters associated with the SCK model that are independent of quencher concentration. Similarly to the method described above, this was achieved by analyzing the decay curves at each value used for the fixed parameter. This method also requires decay curves to be measured over a sufficiently wide range of quencher concentrations. Upper and lower limits for the correct value of the fixed parameter could be determined, but, as with the approach described above, the precise values for the three fundamental parameters associated with the SCK model could not be determined unambiguously.

Other authors have also attempted the analysis of fluorescence decay curves using the expression for $k(t)$ obtained from the SCK model. The approach used by Eads *et al.* [8,11] was to fix the value for the mutual diffusion coefficient D at a value equal to the sum of the bulk diffusion coefficients of the two reactants. In this particular case, one of these diffusion coefficients was measured and the other was estimated using theory and by comparison with similarly shaped molecules. The reaction distance R was constrained to lie within a limited arbitrary range of values estimated by these authors to be that most likely for the contact distance. It can only be presumed that the value of δ was incorporated into this method of analysis as a freely adjustable parameter, because these authors make no reference to the handling of this highly critical parameter. Moreover, the basis for the justification for the use of this methodology,

such as that which can be obtained from the analysis of synthesized fluorescence decay data, was not presented. Consequently, it is difficult to assess whether the relatively poor fitting that was obtained upon analysis of TCSPC decay curves measured using the SCK model is due entirely to the breakdown of the SCK model, as claimed by these authors, rather than being an artifact of the method of analysis.

Nishikawa *et al.* [13] also reported the existence of local minima for the nonlinear least-squares analysis using the SCK model of fluorescence decay curves measured using TCSPC. They attribute this mainly to the sensitivity of the values recovered for the fitted parameters to the value of δ . The approach used by these authors to circumvent this difficulty was to fix the values of R and D such that the steady-state rate constant for diffusion $4\pi RDN$ was equal to the value of the apparent quenching rate constant k_q obtained from steady-state fluorescence quenching measurements. These authors also failed to specify how the value of δ was incorporated in their method of analysis. The equality between $4\pi RDN$ and k_q assumed by these authors only exists in the limit $k_{act} \gg 4\pi RDN$, in which case the reaction is completely diffusion *controlled* and the measured non-exponential fluorescence decay curves can be modeled using the simpler function associated with the Smoluchowski model. In this situation it is unlikely that physically realistic values for k_{act} can be recovered from the analysis of fluorescence decay curves. There seems to be little justification for the assumption of equality between $4\pi RDN$ and k_q used by these authors, undermining the reliability of their reported values for k_{act} .

A relatively simple, alternative methodology to those mentioned above is required in order to obtain directly values for the intrinsic parameters associated with the SCK model from nonlinear least-squares analysis of a fluorescence decay curve measured using the technique of TCSPC. It is demonstrated in this work that such a direct method of analysis is possible, where by “direct” we mean that all three of the fundamental parameters associated with the SCK model are permitted to vary freely. However, the analysis is successful only under limited, but attainable, experimental conditions. The conditions required for enabling distinction to be made between the SCK model and its long-time approximation on the basis of the results of analysis of fluorescence decay curves are also assessed.

THEORETICAL BACKGROUND

The time-dependent rate coefficient for the quenching of an electronically excited fluorophore M^* by a

quenching species Q is given by the following equation, which is derived from the Smoluchowski equation [26] subject to the Collins–Kimball boundary condition [27] (SCK model), and applies to the situation in which either M^* and/or Q have no effective charge:

$$k(t) = a + b \exp(c^2 t) \operatorname{erfc}(ct^{1/2}) \quad (1)$$

where

$$\begin{aligned} a &= k_{act} [1 + (k_{act}/4\pi RDN)]^{-1} \\ b &= k_{act} [1 + (4\pi RDN/k_{act})]^{-1} \\ c &= \{[1 + (k_{act}/4\pi RDN)]D^{1/2}\}/R \end{aligned}$$

In the above equations, R is the distance required for reaction between species M^* and Q , D is the sum of their diffusion coefficients, N is Avogadro’s number, and k_{act} is the rate constant for the intrinsic bimolecular reaction at the reaction distance R . The values for the parameters R , D , and k_{act} can be calculated by using the following equations [6,10]:

$$\begin{aligned} R^3 &= \{a^2 [1 + (b/a)]^3\}/(4\pi bc^2) \quad (2) \\ D &= [a(a+b)]/(4\pi Rb) \quad (3) \\ k_{act} &= a + b \quad (4) \end{aligned}$$

The time dependence of the concentration of electronically excited M molecules M^* in the presence of quencher Q is given by

$$\frac{d[M^*]_t}{dt} = -[M^*]_t(\tau_0^{-1} + k(t)[Q]) \quad (5)$$

where τ_0 is the unquenched fluorescence lifetime of M^* , $[M^*]_t$ is the concentration of M^* at time t , and $[Q]$ is the concentration of quencher. Equation (6) is the function that is predicted for the time-dependent decay of fluorescence intensity $F(t)$ according to the SCK model and is obtained upon substitution of Eq. (1) for $k(t)$ in Eq. (5), and then integration [6,7,9,10,12]

$$F(t) = F(0) \exp\left\{-\left(\tau_0^{-1} + p\right)t - \frac{q}{c^2} \left[\exp(c^2 t) \operatorname{erfc}(ct^{1/2}) - 1 + \frac{2ct^{1/2}}{\pi^{1/2}} \right]\right\} \quad (6)$$

where $p = a[Q]$, $q = b[Q]$, and the units of p , q , and c are ns^{-1} , ns^{-1} , and $\text{ns}^{-1/2}$, respectively, and $F(0)$ is the fluorescence intensity at $t = 0$.

At sufficiently long times Eq. (1) reduces to the following equation, which has the same functional form as the equation predicted by the Smoluchowski model [1,7,11,12,19,25]:

$$k(t) = 4\pi R'DN \left[1 + \frac{R'}{(\pi Dt)^{1/2}} \right] \quad (7)$$

where

$$\frac{1}{R'} = \frac{1}{R} + \frac{4\pi DN}{k_{\text{act}}} \quad (8)$$

The equation for $k(t)$ that is predicted by the Smoluchowski model is obtained in the limit as $k_{\text{act}} \rightarrow \infty$, in which case $R' = R$. The solution to Eq. (5) that is obtained upon substitution of Eq. (7) for $k(t)$ and then integration is given by

$$F(t) = F(0) \exp(-At - Bt^{1/2}) \quad (9)$$

where

$$A = \tau_0^{-1} + 4\pi R'DN[Q]$$

$$B = 8(\pi D)^{1/2} N[Q](R')^2$$

Periasamy *et al.* [9,10] obtained an expression for $F(t)$ that is equivalent to that shown in Eq. (9) by applying the following approximations to Eq. (6):

$$\left. \begin{aligned} \exp(c^2t) \operatorname{erfc}(ct^{1/2}) &\approx \frac{1}{ct^{1/2}\pi} \\ \frac{2ct^{1/2}}{\pi^{1/2}} &\gg \frac{1}{ct^{1/2}\pi^{1/2}} \end{aligned} \right\} \text{for } ct^{1/2} \gg 7$$

The expression derived by these authors for $F(t)$ is

$$F(t) = G(0) \exp(-At - Bt^{1/2}) \quad (10)$$

where

$$G(0) = F(0) \exp\left(\frac{q}{c^2}\right)$$

METHODS

Synthesis of Fluorescence Decay Data

Two different instrument response functions $L(t)$ were used in this work. One response function having a full-width at half-minimum (fwhm) of 37 ps and 1.0×10^4 counts in the channel of maximum intensity (CMI) was generated as described previously [28]. The other was measured using a flash-lamp TCSPC system and has a fwhm of approximately 2.8 ns. These response functions were normalized to unit area before being used to calculate the fluorescence decay curves which were synthesized for measurements using the technique of TCSPC. The time interval per channel for the 37-ps

fwhm response function was 4.9 ps and that for the flash-lamp instrument response function was 107 ps.

The fluorescence decay data $I(t)$ were calculated by using the convolution integral

$$I(t) = \int_0^t F(t-t')L(t'-\delta) dt' \quad (11)$$

where $F(t)$ is the value of the decay function shown in Eq. (6) at time t , and t' is a dummy time variable. A value for the time-zero shift parameter δ of zero channels was used for the calculation of all the decay curves synthesized in this work.

The function introduced by Press *et al.* [30] was used to approximate the term involving the exponentially scaled complementary error function $\exp(c^2t) \operatorname{erfc}(ct^{1/2})$ in Eq. (6), as recommended by Das and Periasamy [7], and this approximation can be expressed as follows:

$$\exp(c^2t) \operatorname{erfc}(ct^{1/2}) \approx T \exp(b_0 + b_1T + b_2T^2 + b_3T^3 + \dots + b_9T^9) \quad (12)$$

where $T = (1 + 0.5ct^{1/2})^{-1}$

and

$$b_0 = -1.26551223, \quad b_1 = 1.00002368, \quad b_2 = 0.037409196$$

$$b_3 = -0.18628806, \quad b_4 = 0.27886807, \quad b_5 = -1.13520398$$

$$b_6 = -1.26551223, \quad b_7 = 1.48851587, \quad b_8 = -0.82215223$$

$$b_9 = 0.17087277$$

Each synthesized decay curve was normalized so as to have a known number of counts in the CMI. Noise with a Gaussian distribution, which approximates closely the Poissonian noise distribution in real fluorescence decay data collected using the TCSPC technique, provided the number of counts exceeds 20, was added to the decay data $I(t)$ by using the procedure described by Demas [31]. Noise was also added to the 37-ps fwhm response function so that each decay curve calculated using this response function contained a noise pattern different from that of the response function. No noise was added to the measured flash-lamp instrument response function.

Analysis of Simulated Fluorescence Decay Data

The synthesized fluorescence decay curves $I(t)$ were analyzed by iterative reconvolution of the response function $L(t)$ with the fitted function $F(t)$. In this method of analysis the fitted parameters are adjusted using an algorithm based on the Levenberg-Marquardt procedure [32-34] in such a way as to minimize the reduced chi-square parameter χ_r^2 , which is defined as the weighted sum of the squares of the deviations of the

experimental data from the calculated fitted function. Values for χ^2 ranging from approximately 0.8 to 1.2 should be obtained if the fitted function is appropriate [32].

The equations for the partial derivatives of the fitted function given by Eq. (6) with respect to the fitted parameters [7] are

$$\frac{\partial F_i}{\partial F(0)} = \frac{F[i]}{F(0)} \quad (13)$$

$$\frac{\partial F_i}{\partial p} = \sum_{j=1}^i F[i-j]L[j-\delta] (-[i-j]) \quad (14)$$

$$\frac{\partial F_i}{\partial q} = \sum_{j=1}^i F[i-j]L[j-\delta] \left(\frac{-1}{c^2}\right) \left[\exp(x^2) \operatorname{erfc}(x)(1-x^2)^{-1} + \frac{2x}{\pi^{1/2}} \right] \quad (15)$$

$$\frac{\partial F_i}{\partial c} = \sum_{j=1}^i F[i-j]L[j-\delta] \left(\frac{2q}{c^2}\right) \left[\exp(x^2) \operatorname{erfc}(x)(1-x^2)^{-1} + \frac{2x}{\pi^{1/2}} \right] \quad (16)$$

$$\frac{\partial F_i}{\partial \delta} = \sum_{j=1}^i F[i-j] \left\{ \frac{L[j-\delta-1] - L[j-\delta+1]}{2} \right\} \quad (17)$$

where $x = c[i-j]^{1/2}$ and the values for the response function intensity at noninteger channel numbers were calculated by linear interpolation. The partial derivative shown in Eq. (17) differs from that used by Das and Periasamy [7].

The search for the minimum in the χ^2 surface was terminated if, for two successive iterations, the fractional change in the value for this parameter and/or the fractional change in the fitted parameters was less than 10^{-3} . The search was also terminated if, after five successive iterations, the value for χ^2 did not decrease. The same initial value and criteria for the variation of λ , the multiplication factor for the modification of the diagonal elements of the Hessian matrix, as those used by Das and Periasamy [7] were adopted for this work.

For the analyses in this work the parameter $F(0)$, which corresponds to the fluorescence intensity at $t = 0$, is a fitted preexponential factor which equals the number of counts in the CMI when the instrument response function is a true delta pulse. The parameter $G(0)$ in Eq. (10) is a constant that is proportional to the preexponential factor and is also predicted to be a function of quencher concentration [9]. Therefore, the recovered value for this parameter will not be equal to the preexponential factor. However, it can be included as an adjustable parameter in the decay curve analysis and, for the purposes of this work, no distinction is made be-

tween the constants $G(0)$ and $F(0)$. Analysis of the synthesized decay curves using Eqs. (6) and (10) was performed using a DEC 5000/200 minicomputer.

RESULTS AND DISCUSSION

Decay Curves Synthesized Using the Flash-Lamp Instrument Response Function

Fluorescence decay curves containing between 1.0×10^4 and 1.0×10^5 counts in the CMI were synthesized using the flash-lamp instrument response function. The values for p , q , and c used for the calculation of these synthesized decay curves were 0.188 ns^{-1} , 0.121 ns^{-1} , and $2.21 \text{ ns}^{-1/2}$, respectively. These values correspond to values for R , D , and k_{act} of 1.52 nm , $4.20 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$, and $3.09 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$, respectively, when the quencher concentration is 0.01 M . These values for R , D , and k_{act} were chosen because they represent the results of a recent study of the fluorescence quenching reaction between 9,10-diphenylanthracene and maleic anhydride in acetonitrile [35]. The value for τ_0 used throughout this work was 8.40 ns . The synthesized decay curve having 1.0×10^4 counts in the CMI is shown in Fig. 1. The results of the analysis of these fluorescence decay curves according to the SCK model [see Eq. (6)] are summarized in Table I.

The five rows of data that are shown in Table I for the analysis of each of the synthesized decay curves are the results of the analysis corresponding to each set of initial guesses (1)–(5) given in footnote *b* to this table. The values recovered for the fitted parameters p , q , and c and the time-zero shift parameter δ from this analysis are highly dependent on the values used for the initial guesses for these parameters, and instabilities in the analysis similar to those reported by Das and Periasamy [7] were observed. The results presented in Table I indicate that reliable values for the fitted parameters cannot be recovered from this type of analysis of fluorescence decay curves measured using the technique of TCSPC for these particular experimental conditions.

The distributions of reduced residuals that were obtained from the analysis of these fluorescence decay curves using an exponential function are shown in Fig. 2. There is no clear deviation from exponentiality observed for the synthesized decay curves having 1.0×10^4 and 2.0×10^4 counts in the CMI. Distinct deviations from exponentiality are only evident for the decay curves containing 5.0×10^4 or 1.0×10^5 counts in the CMI. The distributions of reduced residuals corresponding to analysis of these same synthesized decay curves

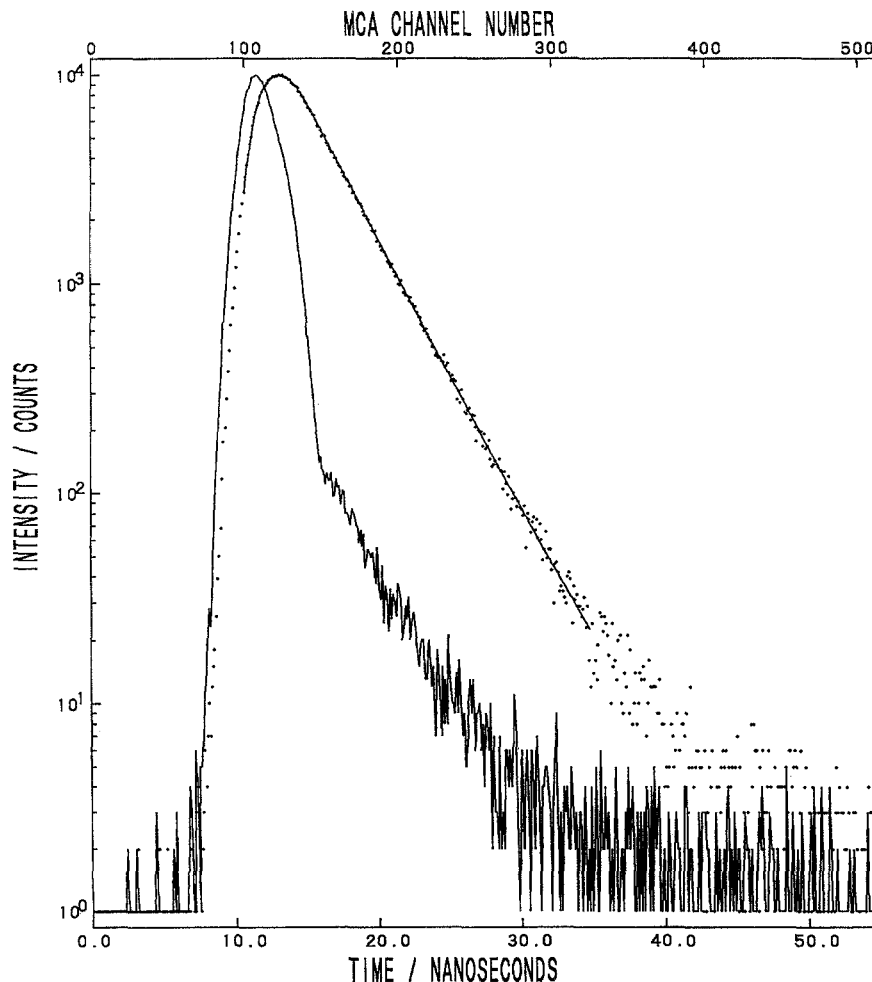


Fig. 1. Fluorescence decay curve (dots) synthesized according to the function derived from the SCK model [Eq. (6)] with $\tau_0 = 8.4$ ns, $R = 1.52$ nm, $D = 4.20 \times 10^{-9}$ m² s⁻¹, and $k_{act} = 3.09 \times 10^{10}$ M⁻¹ s⁻¹ at a quencher concentration of 0.01 M. The solid line through the dots is the best-fit decay curve obtained from analysis using Eq. (10). The other solid line is an instrument response function measured for a flash-lamp TCSPC apparatus (fwhm = 2.8 ns, channel width = 107 ps).

using the long-time approximation [Eq. (10)] are shown in Fig. 3. The quality of fitting in all cases is excellent. The values recovered for R' and D were independent of the initial guesses and are presented in Table II for each synthesized decay curve. The values recovered for R' are in very good agreement with the value for R' of 0.59 nm which was calculated using Eq. (8). The values recovered for D are also in very good agreement with the value of 4.20×10^{-9} m² s⁻¹ which was used in the synthesis of the fluorescence decay curves.

These results demonstrate that analysis of fluorescence decay curves using the long-time approximation to the SCK model is justified for measurements performed using this time scale for reactions having values for R , D , and k_{act} of magnitude comparable with those used in this work. The inequality between the recovered

values for R' and the value for R used to synthesize the decay curves illustrates the inadequacy of the Smoluchowski model as a description of the kinetics in this case. This is a result of the value used for k_{act} being finite. In fact, the value used for k_{act} in this work is smaller than $4\pi RDN$, but still large enough so as to ensure that the time dependence of the rate of reaction can be detected.

The error that is introduced by using the long-time approximation (LTA) to the SCK model [Eq. (10)] instead of the SCK model itself [Eq. (6)] for decay curve analysis can be defined as [10]

$$\text{error} = \frac{I_{LTA} - I_{SCK}}{I_{SCK}} \quad (18)$$

Table I. Results from the Analysis of Synthesized Fluorescence Decay Curves Convolved with the Flash-Lamp Response Function Using the SCK Model^a

| $I_{\max}/10^4$ (counts) | Initial guess ^b | p (ns^{-1}) | q (ns^{-1}) | c ($\text{ns}^{-1/2}$) | δ (channels) | χ_r^2 |
|-----------------------------|-------------------------------|-----------------------------|-----------------------------|-------------------------------|------------------------|------------|
| 1.0 | 1 | 0.189 | 0.414 | 8.08 | 0.0641 | 0.9446 |
| | 2 | 0.189 | 65.3 | 1283 | 0.0708 | 0.9445 |
| | 3 | 0.189 | 7.10 | 139 | 0.0704 | 0.9445 |
| | 4 | 0.189 | 50.4 | 991 | 0.0707 | 0.9445 |
| | 5 | 0.189 | 59.2 | 1165 | 0.0706 | 0.9445 |
| 2.0 | 1 | 0.193 | 0.351 | 9.62 | -0.0703 | 1.1486 |
| | 2 | 0.193 | 118 | 3264 | -0.0665 | 1.1486 |
| | 3 | 0.195 | 5.47 | 194 | -0.1088 | 1.1598 |
| | 4 | 0.193 | 41.9 | 1155 | -0.0665 | 1.1486 |
| | 5 | 0.193 | 48.7 | 1343 | -0.0666 | 1.1486 |
| 5.0 | 1 | 0.191 | 0.374 | 9.26 | -0.0159 | 1.1176 |
| | 2 | 0.191 | 50.3 | 1251 | -0.0115 | 1.1173 |
| | 3 | 0.191 | 5.91 | 147 | -0.0118 | 1.1173 |
| | 4 | 0.191 | 43.3 | 1078 | -0.0116 | 1.1173 |
| | 5 | 0.191 | 51.6 | 1285 | -0.0116 | 1.1173 |
| 10.0 | 1 | 0.189 | 0.404 | 8.40 | -0.0013 | 0.8740 |
| | 2 | 0.189 | 127 | 2650 | 0.0047 | 0.8738 |
| | 3 | 0.189 | 6.73 | 141 | 0.0043 | 0.8738 |
| | 4 | 0.189 | 48.1 | 1005 | 0.0047 | 0.8738 |
| | 5 | 0.189 | 56.5 | 1180 | 0.0046 | 0.8738 |

^aParameters used for decay curve synthesis: $p = 0.188 \text{ ns}^{-1}$, $q = 0.121 \text{ ns}^{-1}$, $c = 2.21 \text{ ns}^{-1/2}$, $\delta = 0.00$ channels.

^bInitial guesses: (1) $p = 0.5 \text{ ns}^{-1}$, $q = 0.5 \text{ ns}^{-1}$, $c = 0.5 \text{ ns}^{-1/2}$, $\delta = 0.00$ channels. (2) $p = 1.0 \text{ ns}^{-1}$, $q = 1.0 \text{ ns}^{-1}$, $c = 1.0 \text{ ns}^{-1/2}$, $\delta = 0.00$ channels. (3) $p = 1.0 \text{ ns}^{-1}$, $q = 2.0 \text{ ns}^{-1}$, $c = 3.0 \text{ ns}^{-1/2}$, $\delta = 0.00$ channels. (4) $p = 1.0 \text{ ns}^{-1}$, $q = 10 \text{ ns}^{-1}$, $c = 30 \text{ ns}^{-1/2}$, $\delta = 0.00$ channels. (5) $p = 1.0 \text{ ns}^{-1}$, $q = 20 \text{ ns}^{-1}$, $c = 40 \text{ ns}^{-1/2}$, $\delta = 0.00$ channels.

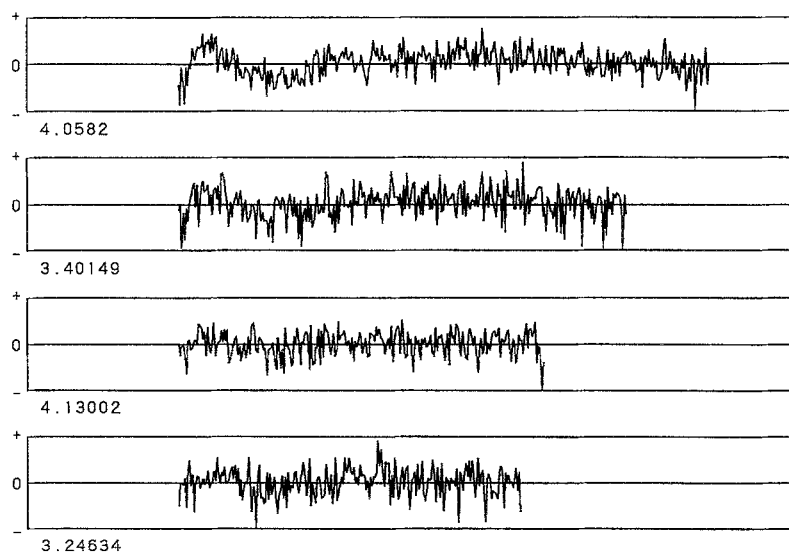


Fig. 2. Distribution of reduced residuals corresponding to the analysis using an exponential function of fluorescence decay curves synthesized using Eq. (6) and convolved with the response function shown in Fig. 1. See text for parameter values used to synthesize these decay curves. The number of counts in the CMI and the values for the reduced chi-square parameter are, from bottom panel to top: 1.0×10^4 , $\chi_r^2 = 1.01$; 2.0×10^4 , $\chi_r^2 = 1.20$; 5.0×10^4 , $\chi_r^2 = 1.31$; 1.0×10^5 , $\chi_r^2 = 1.32$.

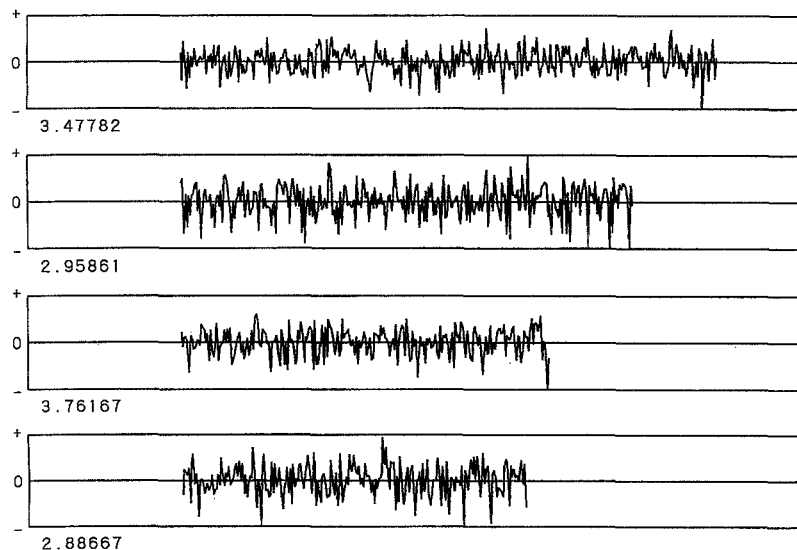


Fig. 3. Distribution of reduced residuals corresponding to the analysis using Eq. (10) of fluorescence decay curves synthesized using Eq. (6) and convoluted with the response function shown in Fig. 1. See text for parameter values used to synthesize these decay curves. The number of counts in the CMI and the values for the reduced chi-square parameter are, from bottom panel to top: 1.0×10^4 , $\chi_r^2 = 0.942$; 2.0×10^4 , $\chi_r^2 = 1.15$; 5.0×10^4 , $\chi_r^2 = 1.09$; 1.0×10^5 , $\chi_r^2 = 0.867$.

Table II. Results from the Analysis of Synthesized Fluorescence Decay Curves Convoluted with the Flash-Lamp Response Function Using the Long-Time Approximation to the SCK Model^{a,b}

| $I_{max}/10^4$ (counts) | R' (nm) | $D/10^9$ (m ² sec ⁻¹) | δ (channels) | χ_r^2 |
|----------------------------|--------------|---|------------------------|------------|
| 1.0 | 0.66 | 3.7 | 0.11 | 0.942 |
| 2.0 | 0.48 | 5.3 | -0.056 | 1.15 |
| 5.0 | 0.54 | 4.6 | 0.016 | 1.09 |
| 10.0 | 0.56 | 4.4 | 0.013 | 0.867 |

^aParameters used for decay curve synthesis: $p = 0.188 \text{ ns}^{-1}$, $q = 0.121 \text{ ns}^{-1}$, $c = 2.21 \text{ ns}^{-1/2}$, $\delta = 0.00$ channels.

^b $R' = 0.59 \text{ nm}$ [from Eq. (8)] and $D = 4.20 \times 10^{-9} \text{ m}^2 \text{ sec}^{-1}$.

The error that is induced by using the long-time approximation to the SCK model to analyze the fluorescence decay curves synthesized using the flash-lamp response function is illustrated in Fig. 4 for the decay curve containing 1.0×10^4 counts in the CMI. This error was calculated by first convoluting the function given in Eq. (6) with the instrument response function. A second decay curve was then synthesized by convoluting the function given by Eq. (10) with the instrument response function. The value of $G(0)$ for this second decay curve was calculated by using the same values for $F(0)$, q , and c as those used for the decay curve synthesized according to Eq. (6). The value for I_{max} shown in this figure refers to the number of counts in the CMI for the decay

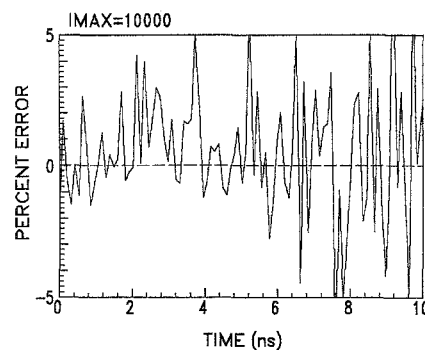


Fig. 4. Plot of error invoked by using Eq. (10) to analyze a decay curve synthesized using Eq. (6) and convoluted with the instrument response function shown in Fig. 1. Here I_{max} refers to the number of counts in the CMI for the decay curve synthesized according to Eq. (6). Error was calculated using Eq. (18).

curve synthesized according to Eq. (6). Noise was then added to each of these decay curves prior to the calculation of error using Eq. (18). The value used for R' in Eq. (10) was obtained from the values of R , D , and k_{act} used to generate the synthesized decay curves. The value for D was the same for the calculation of both decay curves. Time zero corresponds to the starting channel for the fitting of the synthesized decay curves.

The plot shown in Fig. 4 indicates that the functions associated with the SCK model and its long-time approximation are essentially indistinguishable for the val-

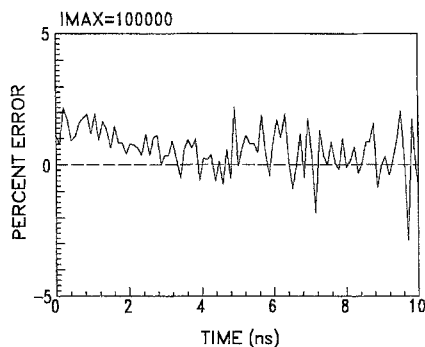


Fig. 5. Plot of error invoked by using Eq. (10) to analyze a decay curve synthesized using Eq. (6) and convoluted with the instrument response function shown in Fig. 1. Here I_{max} refers to the number of counts in the CMI for the decay curve synthesized according to Eq. (6). Error was calculated using Eq. (18).

ues of p , q , and c used in this work. Consequently, it would be expected that discrimination between the SCK model and its long-time approximation would be impossible based on the analysis of this fluorescence decay curve. As noted above, the results of analysis of this decay curve using an exponential function suggest that this decay curve is actually exponential. The apparent success of the exponential function in fitting this decay curve, which was synthesized using the SCK function, can be attributed to the optimization of the preexponential factor and δ so as to minimize the value of χ^2 . The effect of the adjustment of these parameters in concealing nonexponentiality in the fluorescence decay curve diminishes as the number of counts in the CMI increases. This is demonstrated by the clear deviation from exponentiality displayed by the decay curve containing 1.0×10^5 counts in the CMI. This example serves to illustrate the extreme care required when interpreting results obtained from the analysis of fluorescence decay curves of relatively low precision.

The error induced by using the long-time approximation for the analysis of the fluorescence decay curve containing 1.0×10^5 counts in the CMI is shown in Fig. 5. This plot demonstrates that the functions given in Eqs. (6) and (10) should, in principle, be distinguishable for this decay curve and this implies that deviation between Eq. (10) and the decay curve synthesized using Eq. (6) should be evident. However, the random distribution of reduced residuals shown in the uppermost panel of Fig. 3 indicates that Eq. (10) provides a statistically satisfactory fit to the synthesized decay curve. This apparent inconsistency again arises from the optimization of the preexponential factor and δ that occurs in attaining a minimum for the value of χ^2 . This results in a minimi-

zation of the small deviation between the SCK model and its long-time approximation shown in Fig. 5.

Decay Curves Synthesized Using the 37-ps fwhm Instrument Response Function

Another set of fluorescence decay curves containing between 1.0×10^4 and 1.0×10^5 counts in the CMI was synthesized using a simulated instrument response function having a fwhm of 37 ps. Instrument response functions of this magnitude are now accessible for TCSPC experiments [13]. The values of the parameters R , D , and k_{act} used for the synthesis of these decay curves were the same as those used for the decay curves synthesized using the flash-lamp response function, but the quencher concentration was increased to 0.1 M. The decay curve having 1.0×10^4 counts in the CMI that was synthesized using this response function is shown in Fig. 6. The distributions of reduced residuals that were obtained upon analysis of these fluorescence decay curves using an exponential function are shown in Fig. 7. The clear deviation from exponentiality of these decay curves is apparent even for the decay curve containing 1.0×10^4 counts in the CMI, in contrast with the decay curve shown in Fig. 1. Evidently, the extent of deviation from exponentiality on the time scale of these decay curves is too large to be obscured by optimization of the preexponential factor and δ .

The distributions of reduced residuals corresponding to analysis of the decay curves using Eq. (10) are shown in Fig. 8. The quality of fitting clearly deteriorates as the number of counts in the CMI increases, becoming unacceptably poor for the decay curve containing 1.0×10^5 counts in the CMI. These results indicate that it is possible to detect the breakdown of the long-time approximation to the SCK model directly by analyzing fluorescence decay curves measured on this time scale using TCSPC. Furthermore, the values recovered for R' and D from this analysis, while being independent of initial guesses, are in poor agreement with those expected from the values of R , D , and k_{act} used for the calculation of the synthesized decay curves. The values recovered for R' and D are summarized in Table III.

The origin of the breakdown of the long-time approximation to the SCK model can be seen from the plots shown in Figs. 9 and 10. The discrepancy between the functions corresponding to the SCK model and its long-time approximation is characterized by a spike that exists for approximately 100 ps. This spike is not resolved in the lower-time-resolution decay curves synthesized using the flash-lamp response function (see Figs. 4 and 5). Clear deviation between the functions given in

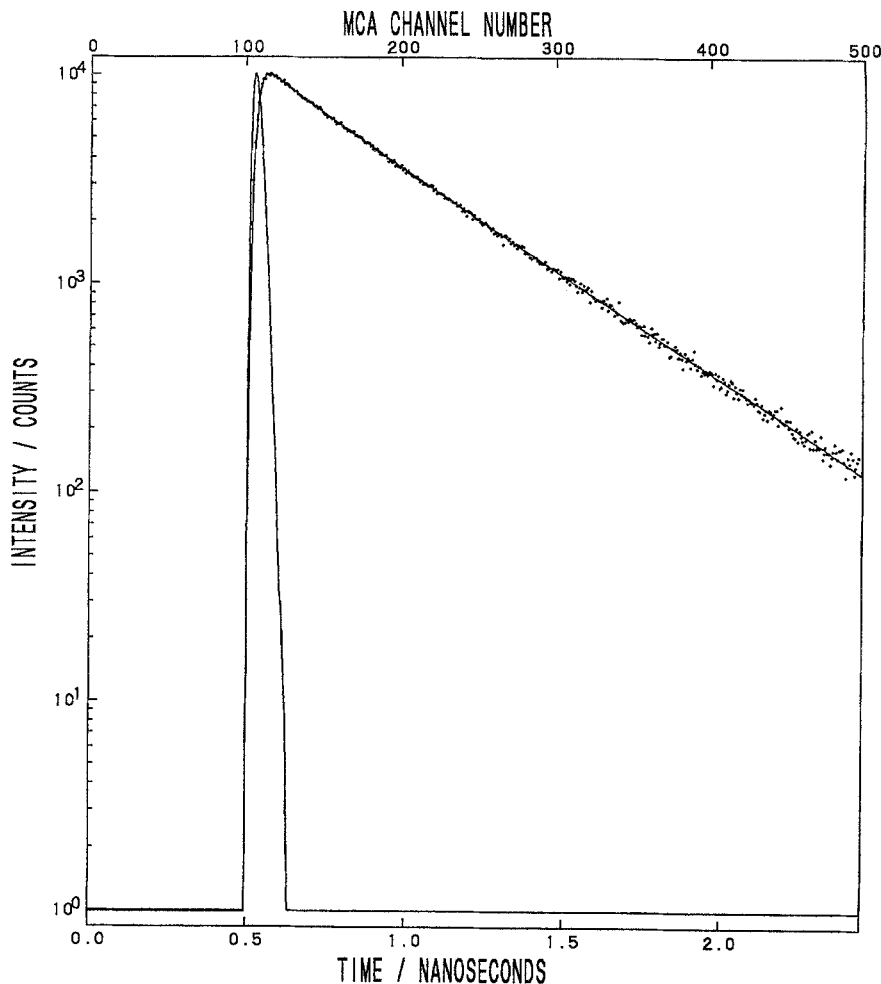


Fig. 6. Fluorescence decay curve (dots) synthesized according to the function derived from the SCK model [Eq. (6)] with $\tau_0 = 8.4$ ns, $R = 1.52$ nm, $D = 4.20 \times 10^{-9}$ m² s⁻¹, and $k_{act} = 3.09 \times 10^{10}$ M⁻¹ s⁻¹ at a quencher concentration of 0.1 M. The solid line through the dots is the best-fit decay curve obtained from analysis using Eq. (10). The other solid line is the instrument response function (fwhm = 37 ps, channel width = 4.9 ps).

Eqs. (6) and (10) is apparent for approximately 1 ns after time zero. The magnitude of this deviation is more significant for the decay curves convoluted with the shorter response function than that for the decay curves synthesized using the flash-lamp response function. This is at least partly a consequence of the difference in the values of q for these two cases, being an order of magnitude larger for the decay curves generated using the narrower response profile due to the higher quencher concentration. This results in a value for the factor in Eq. (10), $G(0)$, that is significantly larger than that for $F(0)$ in Eq. (6). Consequently, the absolute magnitude of the percent error quoted in these plots cannot be compared directly for the decay curves synthesized using the different response functions.

The extent of the deviation shown in Figs. 9 and 10 is exaggerated due to the difference in magnitude between $F(0)$ and $G(0)$ used for the calculation of these plots. This results in the number of counts in the CMI being dependent upon whether the decay curve was synthesized using Eq. (6) or Eq. (10). The magnitude of the deviation in these plots, therefore, does not necessarily correspond to the actual deviation that would be detected upon analysis of a fluorescence decay curve. The deviation between the two functions will be further reduced in the analysis of the synthesized decay curves due to the effects of optimization of the preexponential factor and δ . The deviation is reduced to such an extent that the apparently successful analysis of the decay curve containing 1.0×10^4 counts in the CMI using Eq. (10)

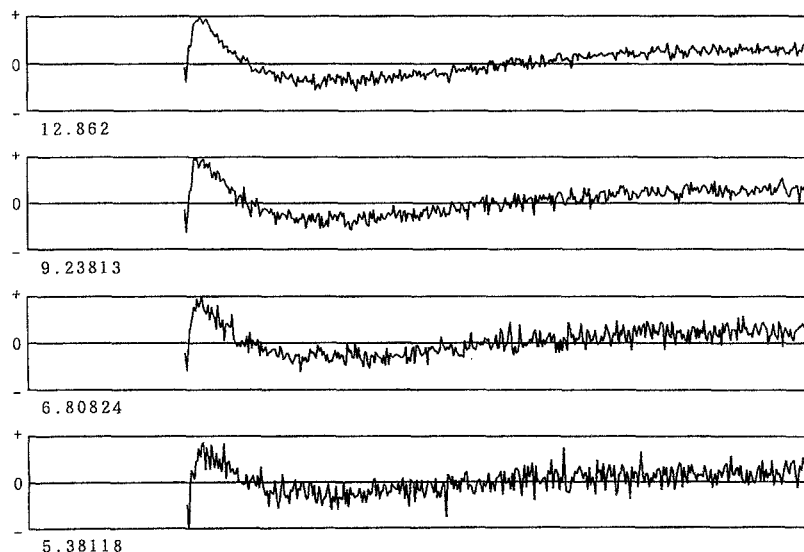


Fig. 7. Distribution of reduced residuals corresponding to the analysis using an exponential function of fluorescence decay curves synthesized using Eq. (6) and convoluted with the 37-ps fwhm response function. See text for parameter values used to synthesize these decay curves. The number of counts in the CMI and the values for the reduced chi-square parameter, are from bottom panel to top: 1.0×10^4 , $\chi_r^2 = 1.70$; 2.0×10^4 , $\chi_r^2 = 2.28$; 5.0×10^4 , $\chi_r^2 = 3.97$; 1.0×10^5 , $\chi_r^2 = 8.37$.

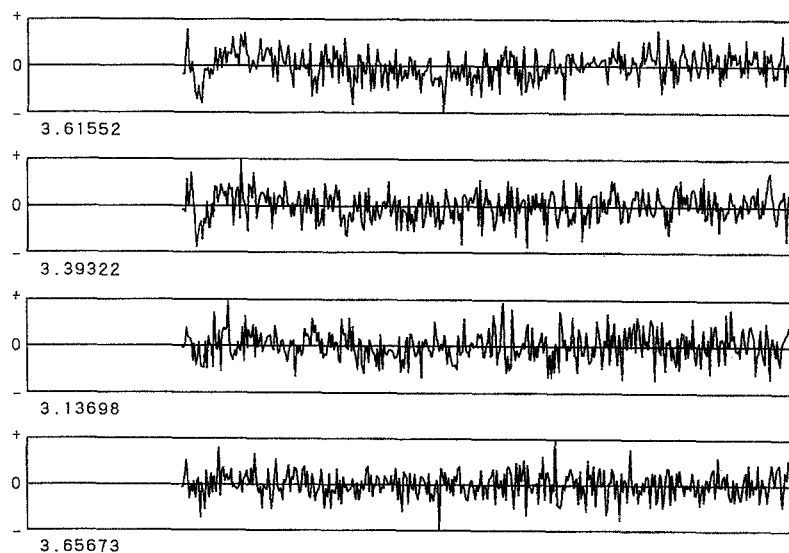


Fig. 8. Distribution of reduced residuals corresponding to the analysis using Eq. (10) of fluorescence decay curves synthesized using Eq. (6) and convoluted with the 37-ps fwhm response function. See text for parameter values used to synthesize these decay curves. The number of counts in the CMI and the values for the reduced chi-square parameter are, from bottom panel to top: 1.0×10^4 , $\chi_r^2 = 1.04$; 2.0×10^4 , $\chi_r^2 = 0.975$; 5.0×10^4 , $\chi_r^2 = 1.10$; 1.0×10^5 , $\chi_r^2 = 1.18$.

would result in incorrect values for R' and D (see Table III). The effectiveness of the optimization of the preexponential factor and δ in obscuring deviations from the long-time approximation decreases as the number of counts in the CMI is increased. The discrepancy between Eq. (6) and Eq. (10) becomes obvious for fluorescence

decay curves containing 5.0×10^4 counts or more in the CMI for the values of the parameters p , q , and c used for the synthesis of these decay curves (see Fig. 8).

The results of the analysis of these synthesized fluorescence decay curves using Eq. (6) are presented in Table IV. The values recovered for the fitted parameters

Table III. Results from the Analysis of Synthesized Fluorescence Decay Curves Convolved with the 37-ps fwhm Response Function Using the Long-Time Approximation to the SCK Model^{a,b}

| $I_{\max}/10^4$ (counts) | R' (nm) | $D/10^9$ (m ² sec ⁻¹) | δ (channels) | χ_r^2 |
|-----------------------------|--------------|---|------------------------|------------|
| 1.0 | 0.39 | 6.9 | 0.084 | 1.04 |
| 2.0 | 0.40 | 6.5 | 0.066 | 0.975 |
| 5.0 | 0.40 | 6.5 | 0.083 | 1.10 |
| 10.0 | 0.39 | 6.7 | 0.068 | 1.18 |

^aParameters used for decay curve synthesis: $p = 1.88 \text{ ns}^{-1}$, $q = 1.21 \text{ ns}^{-1}$, $c = 2.21 \text{ ns}^{-1/2}$, $\delta = 0.00$ channels.

^b $R' = 0.59 \text{ nm}$ [from Eq. (8)] and $D = 4.20 \times 10^{-9} \text{ m}^2 \text{ sec}^{-1}$.

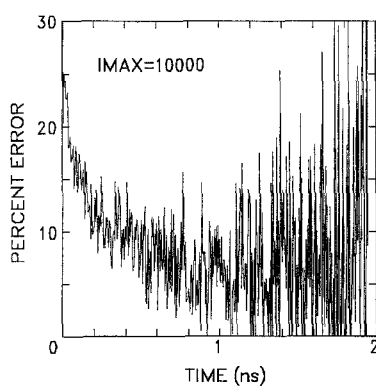


Fig. 9. Plot of error invoked by using Eq. (10) to analyze a decay curve synthesized using Eq. (6) and convolved with the 37-ps fwhm instrument response function. I_{\max} refers to the number of counts in the CMI for the decay curve synthesized according to Eq. (6). Error was calculated using Eq. (18).

are highly dependent upon initial guesses for the decay curves containing 1.0×10^4 and 2.0×10^4 counts in the CMI. This corresponds with the statistically successful fits obtained using Eq. (10) to analyze these decay curves. Analysis of the decay curves containing 5.0×10^4 and 1.0×10^5 counts in the CMI yields values for the fitted parameters that are in excellent agreement with those used for the generation of the synthesized decay curves. It is also seen from the upper two panels in Fig. 8 that these decay curves display an obvious deviation from the long-time approximation to the SCK model. The distributions of reduced residuals obtained upon analyzing the decay curve containing 1.0×10^5 counts in the CMI using an exponential function and the functions associated with the SCK model and its long-time approximation are shown for comparison in Fig. 11. The significant improvement in the quality of fitting obtained by analysis using the SCK function compared with that

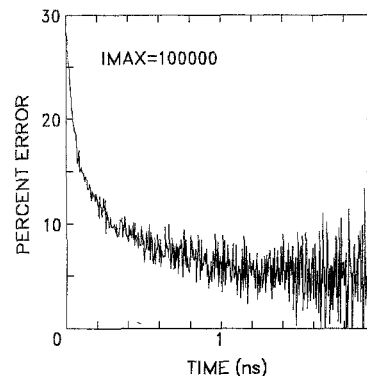


Fig. 10. Plot of error invoked by using Eq. (10) to analyze a decay curve synthesized using Eq. (6) and convolved with the 37-ps fwhm instrument response function. I_{\max} refers to the number of counts in the CMI for the decay curve synthesized according to Eq. (6). Error was calculated using Eq. (18).

for the long-time approximation is illustrated clearly by the plots in this figure.

Several publications [8,11–13,24,25] have appeared recently in which the decay of fluorescence for bimolecular fluorescence quenching reactions in solution involving intermolecular electron transfer has been measured over a wide range of time resolution using TCSPC, and these decay curves have been analyzed using the SCK model. It has been proposed recently [24,25,36] that the redox couple will diffuse to some “optimized” separation distance, which is often greater than the contact distance, at which the rate constant for electron transfer is maximized. This optimized separation distance depends upon the free energy involved in the electron-transfer reaction. The recovery of the values for R , D , and k_{act} at this optimized distance should be possible from the direct analysis of a fluorescence decay curve measured using TCSPC according to the SCK model subject to the following conditions: (a) the fluorescence decay measurement is performed using a time scale that is sufficiently long so that the reaction is affected by the time-dependent effects of translational diffusion of the reactants, (b) the time scale is sufficiently short and the decay data are of sufficiently high precision so as to enable the detection of deviations between the SCK model and its long-time approximation. Fluorescence decay curve measurements performed using ultrashort time scales probe those reactions occurring so rapidly that the effects of diffusion in typical solvents at room temperature are insignificant. The fluorescence decay curves obtained from these measurements are more likely to display nonexponentiality as a result of the dis-

Table IV. Results from the Analysis of Synthesized Fluorescence Decay Curves Convolved with the 37-ps fwhm Response Function Using the SCK Model^a

| $I_{\max}/10^4$ (counts) | Initial guess ^b | p (ns ⁻¹) | q (ns ⁻¹) | c (ns ^{-1/2}) | δ (channels) | χ^2 |
|-----------------------------|-------------------------------|----------------------------|----------------------------|------------------------------|------------------------|----------|
| 1.0 | 1 | 1.90 | 1.15 | 2.22 | 0.0048 | 1.018 |
| | 2 | 1.26 | 1.39 | 0.432 | -0.0054 | 1.053 |
| | 3 | 1.91 | 1.19 | 2.41 | 0.0088 | 1.017 |
| | 4 | 1.92 | 1.24 | 2.61 | 0.0175 | 1.016 |
| | 5 | 2.01 | 10.4 | 35.2 | 0.0690 | 1.038 |
| 2.0 | 1 | 1.30 | 1.39 | 0.502 | -0.0050 | 1.025 |
| | 2 | 1.23 | 1.45 | 0.458 | -0.0033 | 1.031 |
| | 3 | 1.91 | 1.36 | 2.86 | -0.0003 | 0.930 |
| | 4 | 1.91 | 1.36 | 2.87 | -0.0001 | 0.930 |
| | 5 | 1.91 | 1.36 | 2.85 | -0.0005 | 0.930 |
| 5.0 | 1 | 1.89 | 1.27 | 2.46 | 0.0063 | 0.973 |
| | 2 | 1.89 | 1.27 | 2.46 | 0.0063 | 0.973 |
| | 3 | 1.89 | 1.27 | 2.47 | 0.0067 | 0.973 |
| | 4 | 1.90 | 1.34 | 2.72 | 0.0169 | 0.975 |
| | 5 | 1.90 | 1.33 | 2.72 | 0.0168 | 0.975 |
| 10.0 | 1 | 1.89 | 1.20 | 2.23 | 0.0006 | 0.923 |
| | 2 | 1.89 | 1.20 | 2.23 | 0.0007 | 0.923 |
| | 3 | 1.89 | 1.20 | 2.23 | 0.0007 | 0.923 |
| | 4 | 1.89 | 1.25 | 2.43 | 0.0099 | 0.922 |
| | 5 | 1.89 | 1.25 | 2.45 | 0.0107 | 0.922 |

^aParameters used for decay curve synthesis: $p = 1.88 \text{ ns}^{-1}$, $q = 1.21 \text{ ns}^{-1}$, $c = 2.21 \text{ ns}^{-1/2}$, $\delta = 0.00$ channels.

^bInitial guesses: (1) $p = 0.5 \text{ ns}^{-1}$, $q = 0.5 \text{ ns}^{-1}$, $c = 0.5 \text{ ns}^{-1/2}$, $\delta = 0.00$ channels. (2) $p = 1.0 \text{ ns}^{-1}$, $q = 1.0 \text{ ns}^{-1}$, $c = 1.0 \text{ ns}^{-1/2}$, $\delta = 0.00$ channels. (3) $p = 1.0 \text{ ns}^{-1}$, $q = 2.0 \text{ ns}^{-1}$, $c = 3.0 \text{ ns}^{-1/2}$, $\delta = 0.00$ channels. (4) $p = 1.0 \text{ ns}^{-1}$, $q = 10 \text{ ns}^{-1}$, $c = 30 \text{ ns}^{-1/2}$, $\delta = 0.00$ channels. (5) $p = 1.0 \text{ ns}^{-1}$, $q = 20 \text{ ns}^{-1}$, $c = 40 \text{ ns}^{-1/2}$, $\delta = 0.00$ channels.

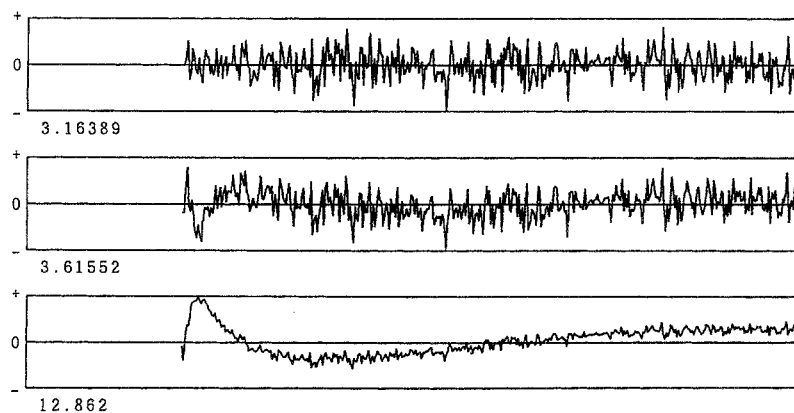


Fig. 11. Plots of reduced residuals corresponding to the analysis of the fluorescence decay curve having 1.0×10^5 counts in the CMI synthesized using Eq. (6) and convoluted with the 37-ps fwhm instrument response function. The lower, middle, and upper panels were obtained from analysis using an exponential function, Eq. (10), and Eq. (6), respectively, and the associated values of the chi-square parameter are $\chi^2 = 8.37$, 1.18, and = 0.922.

tance-dependent nature of the rate constant for the intrinsic bimolecular reaction rather than the effects of diffusion. Under these circumstances analysis of fluorescence decay curves using the SCK model is inappropriate.

CONCLUSIONS

The results obtained in this work demonstrate that it is possible to analyze directly a fluorescence decay

curve measured using the technique of TCSPC according to the SCK model and recover reliable values for R , D , and k_{act} without resorting to the imposition of unnecessary artificial constraints on the values of the fitted parameters. The only provisos are that the decay curve be of sufficiently high precision and be measured using sufficiently high time resolution. Clear deviation between the best-fit curve obtained from analysis using the long-time approximation to the SCK model and the measured fluorescence decay curve will also be observed from a plot of the distribution of reduced residuals for these experimental conditions. The results of this work suggest that a fluorescence decay curve containing approximately 5.0×10^4 counts or more in the CMI and measured using an instrument response function less than approximately 100-ps fwhm should be sufficient to satisfy these experimental requirements.

It was also demonstrated that reliable values for R' and D can be recovered from analysis of fluorescence decay curves measured using lower time resolution, such as those measured using a flash-lamp TCSPC apparatus, by applying the function associated with the long-time approximation to the SCK model. Analysis using this function is more stable and can be used as an alternative to the direct analysis using the SCK model when one of the fundamental parameters R , D , or k_{act} can be varied in some systematic fashion. The parameter D is usually the most amenable to this type of approach through the variation of solvent viscosity. The advantage of direct analysis of decay curves using the decay function associated with the SCK model is that, in principle, it enables values for these parameters to be recovered from analysis of a single fluorescence decay curve measurement.

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