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Photophysics of ethidium bromide complexed to ct-DNA: a maximum entropy study

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Abstract

Time-integrated and time-resolved fluorescence spectroscopies have been used to probe the photophysical properties of ethidium bromide (Eb) complexed to calf thymus DNA (ct-DNA). Fluorescence decay profiles are obtained using the technique of time-correlated single photon counting (TCSPC), and subsequently analysed using conventional sum-of-exponential (SOE) routines and also the maximum entropy method (MEM). Through use of these methods and simulated decay data, it is demonstrated that the kinetics of Eb in the presence of ds-DNA are best described by a generic model consisting of three exponential terms. At all DNA:Eb ratios and NaCl concentrations studied, free Eb is detected. Furthermore, Eb is found to interact with ds-DNA through two mechanisms, each distinguishable by its fluorescence decaytime. Eb is shown to interact with DNA through classic intercalation, and also through binding at secondary sites. The component decaytimes are shown to be a function of NaCl concentration but independent of DNA:Eb molar ratio. © 1998 Elsevier Science B.V.

Keywords: DNA; Intercalation; Ethidium bromide; Maximum entropy method; Fluorescence lifetime

1. Introduction

The term intercalation was first introduced in 1961 to describe the insertion of planar aromatic (or heteroaromatic) compounds between adjacent nucleotide base pairs of double stranded DNA (ds-DNA) [1]. Intercalative binding follows the *neighbour exclusion principle* where every second site along the helix remains unoccupied. The fluorescent dye ethidium bromide (Eb) is of special interest as it demonstrates classic intercalative behaviour [2,3]. In addition, its binding properties are analogous to many anti-neoplastic agents such as those used in chemotherapy. Consequently, the study of DNA/Eb interactions can act as a useful model in the study of binding mechanisms of drug molecules with DNA [4].

In recent years DNA/Eb systems have been analysed using Fluorescence Lifetime Analysis (FLA), and in particular using the technique of time correlated single photon counting (TCSPC) [5–8]. TCSPC

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allows fluorescence lifetimes to be measured on a picosecond time scale with high precision [9]. A large increase in the fluorescence intensity and fluorescence lifetime (τ_f) of Eb as it intercalates with DNA has been demonstrated [10–15]. One of the most recent studies is that of Heller and Greenstock [5]. Their work demonstrates a free solution decaytime of under 2 ns. This increases to between 16 and 23 ns when bound to short strands of ds-DNA. The increase in the fluorescence lifetime is accompanied by an increase in the time-integrated fluorescence intensity.

The low fluorescence intensity and short fluorescence lifetime of Eb in free aqueous solution can be attributed to efficient quenching of the excited state by the transfer of a proton to a solvent (water) molecule [8,16]. However, when Eb binds or intercalates to ds-DNA it is shielded, to some extent, from solvent molecules. Consequently, the rate of excited state quenching decreases, resulting in both an increase in τ_f and an increase in fluorescence quantum yield (ϕ_f).

It has been reported that in addition to conventional intercalation there may also be secondary binding of Eb through an external site (most likely along the phosphate backbone) [17,18]. This secondary site binding is believed to occur at low ionic concentrations (< 0.1 M), after binding at the primary site has been saturated. However, the true nature of the secondary binding is still poorly understood. Accordingly, it is noted that a precise understanding of the fluorescence decaytime variations as Eb interacts with DNA may permit the development of assays able to perform exact DNA quantitation.

The conventional method of recovering decaytimes from a fluorescence decay curve is through the use of iterative, forward convolution methods [19]. The basic principle behind these being the a prori prediction of a specific functional form, normally a sum-of-exponential (SOE) model, for the measured decay. This functional form is transformed into a quantised dataset, and convoluted with the measured instrument response. Subsequent comparison of this convolution with the measured decay is used to evaluate the applicability of the SOE function. This process is repeated, in an iterative manner, until the predicted function most closely approximates the measured decay. At this point decaytimes and amplitudes are extracted. Analysis of DNA/Eb systems to date, has been performed assuming that only one type of binding or intercalation occurs. Hence, fluorescence decays are analysed by fitting the data to a kinetic model based on the sum of two discrete exponential decays. The first representing the decay of the free dye and the second representing the decay of the bound or intercalated dye. Such a method assumes a kinetic model of the system prior to the analysis of the fluorescence decay. Consequently, a bias is introduced at the start of the analysis, and the following questions must be asked.

1. Is the original theoretical model used appropriate?
2. If the optimised parameters give a good statistical description of the system, can they be related to physical parameters?

The implication is that a satisfactory statistical fit of TCSPC data to a SOE kinetic model may provide no true physical description of the system. Studies on the validity of TCSPC analysis protocols by Ware and de Mello have suggested that the SOE approach is ill-conditioned and in many situations can hide underlying complexities (notably when TCSPC data is of low precision) [20–22]. Lifetime distribution analysis (LDA) provides a significant improvement on existing fitting routines. It allows the differentiation of systems containing discrete components from systems made up from a distribution of emitting species. LDA techniques make no a priori assumptions about the physical nature of the system [22,23]. Accordingly no bias is introduced into the analysis. LDA will only display structure that is demanded by the data and the result is therefore independent of any initial guess. LDA produces a kinetic distribution representative of the system, that has a true physical meaning.

In this paper kinetic models previously used to describe Eb decay kinetics are critically assessed, and the complexity of the DNA/Eb system probed. Fluorescence decays of all DNA/Eb solutions are collected using the same TCSPC protocols used in Ref. [5]. However, analysis involves the combination of conventional iterative re-convolution methods, LDA (more specifically the maximum entropy method (MEM)) and simulated decay data to elucidate the true nature of the DNA/Eb system. In

addition, TCSPC data is collected to higher precision to verify the validity of the fitted functions.

2. Materials

Eb (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide, Sigma) showed only a single spot upon TLC (4:1:1 butanol–acetic acid–water) and was used without further purification. Stock solutions of Eb were prepared at 1 mM in distilled water and at two concentrations of NaCl (0.05 M and 1 M). Solutions were stored in the dark below 5°C. Stock solutions of calf thymus (ct) DNA (Sigma type I sodium salt, highly polymerised) were prepared at 1 mg per ml in a pH 7.5 (physiological pH) tris–HCl buffer; again at the two salt concentrations. DNA solutions were left to equilibrate at 5°C in the dark for 48 h before use and then stored under the same conditions. In each experiment the final concentration of DNA was 50 μg/ml or 143 μM (effective nucleotide concentration). Concentrations of Eb were varied from 3 μM to 400 μM, giving an DNA:Eb molar ratio of between 0.36 and 47.7.

3. Experimental

Steady state fluorescence spectra were collected on a Hitachi F4500 fluorescence spectrometer. All spectra were collected and analysed using the Hitachi F4500 software. Excitation and emission slits were set at 2.5 nm. Standard 10 mm path length fused silica fluorescence cuvettes were used for all measurements.

Absorption spectra were measured using a diode array spectrometer Hewlett Packard 8452A, again using 10 mm path length cells. Cuvettes and glassware were cleaned prior to use, by soaking in concentrated nitric acid for 24 h and then washing with copious amounts of distilled water.

Fluorescence decays were collected using the Edinburgh Instruments 199 Fluorescence Lifetime Spectrometer. Excitation and the emission slits were set to 5 mm in all experiments. The flash lamp used was the Edinburgh Instruments 199A Nanosecond Flash Lamp

The lamp was operated at 25 KHz, 8.0 kV and at 1.5 bar of nitrogen. The decay profiles were collected over 512 channels, with a time per channel of 168 ps, until 20 000 counts were collected in the channel of maximum intensity (c_{max}). The instrument response function (IRF) was obtained (fwhm 1.5 ns) using a scattering solution (LUDOX, Sigma). For all experiments the excitation and emission monochromators were set to 337 nm and 580 nm respectively. All measurements were made at 25°C. The system was calibrated by measuring the fluorescence lifetimes of two reference compounds; acridine orange in ethanol ($\tau_{f,exp} = 3.14$ ns), and fluorescein in ethanol ($\tau_{f,exp} = 4.7$ ns). Measured lifetimes are consistent with those in the literature.

4. Data analysis

Initially each decay was analysed as a sum of either one, two or three exponential terms, using a normal iterative, re-convolution routine based on the Levenberg–Marquardt algorithm [24]. Both pre-exponentials and lifetimes are variable parameters of the fitting routine.

Synthetic decay curves were generated using the impulse decay function written in Eq. (1) [25].

$$D(t) = \sum_{i=1}^{N} a(i)\exp[-t/\tau(i)] \quad (1)$$

Here $D(t)$ is the decay intensity at time t, $a(i)$ is the pre-exponential term assigned to the lifetime $\tau(i)$ (both of which are recovered from the SOE analysis), and N is the number of exponentials describing the decay. An experimentally determined IRF was subsequently used to generate a convoluted decay curve $C(t)$.

$$C(t) = \int_{i=1}^{\infty} f(s)\, D(t-s)\,\mathrm{d}s \quad (2)$$

where $f(s)$ is the IRF. Simulated decays were scaled to the same c_{max} as the experimental data, and Gaussian noise added to approximate Poisson counting statistics.

Both the experimental and the simulated decays were analysed using the MEM. This method assumes that the decay is described by 100 exponentials with fixed, logarithmically spaced lifetimes, between 100 and 35 000 ps.

$$I(t) = \sum_{i=1}^{100} a_i \exp(-t/\tau_i) \quad (3)$$

Reconstruction of the amplitudes (a_i) is achieved by maximising the 'entropy like' function,

$$s = -\sum_{i-1}^{100} a_i \ln\left(\frac{a_i}{\sum_{i-1}^{100} a_i}\right) \quad (4)$$

5. Results

5.1. Time integrated spectroscopy

The peak absorbance and λ_{max} of Eb complexed to ds-DNA as a function of DNA:Eb molar ratios are shown in Fig. 1 (for the 1 M NaCl solution). A decrease in the absorbance as the DNA:Eb ratio increases demonstrates the decreasing dye concentration. In addition, when the DNA:Eb molar ratio varies between 1.4 and 14.4, the λ_{max} shifts from 520 to 480 nm. This shift is indicative of the formation of specific complexes between the dye and the DNA [12]. The formation of such complexes causes a shift in the absorption spectrum due to the stabilisation of the excited state upon binding [4]. This shift is only observed in the high DNA:Eb ratio samples since an excess of free Eb normally masks the absorbance contribution from the bound Eb.

Time-integrated fluorescence intensity as a function of DNA:Eb molar ratio is plotted in Fig. 2. An

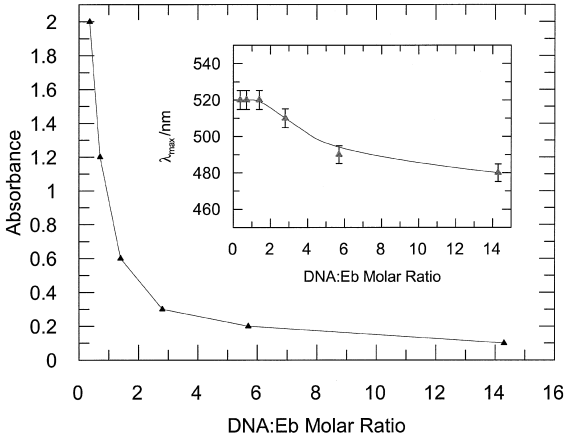

Fig. 1. Peak absorbance as a function of DNA:Eb molar ratio for 1M NaCl solutions. λ_{max} as a function of DNA:Eb molar ratio is also shown (inset).

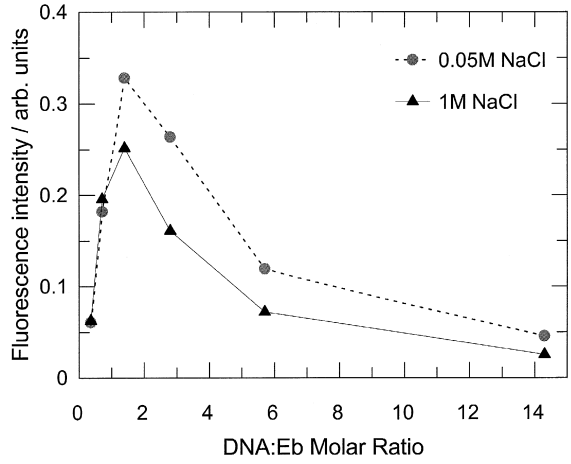

Fig. 2. Time-integrated fluorescence intensity as a function of DNA/Eb molar ratio.

increase in the emission intensity is observed as the DNA:Eb ratio decreases from 14.4 to 1.4. This is a result of dye binding to additional sites along the DNA backbone. As the DNA:Eb ratio drops from 1.4 to 0.36 a reduction in the emission intensity is observed. This reduction may be explained by energy transfer from Eb molecules bound in highly fluorescent sites to molecules bound at secondary sites, which until now have been thought non-fluorescent [12]. The reduction in emission intensity may also be due in part, to the action of free dye on the gross DNA structure. It has been reported that in the presence of high concentrations of bound dye DNA may change its structure, resulting in greater solvent accessibility to the intercalated Eb [8]. This will subsequently lead to the observed reduction in ϕ_f. At all DNA:Eb molar ratios the emission intensity of the 0.05 M salt solutions is greater than that of the 1 M salt solutions. This indicates a greater degree of bound Eb at the lower salt concentration.

5.2. Time resolved spectroscopy

The bulk fluorescence decay profile of Eb in aqueous solution (0.05 M NaCl, pH 7.5) was analysed using a single exponential decay function, and yielded excellent fits at all levels of precision ($\tau_f =$ 1.99 ns, $c_{max} = 5 \times 10^3 - 3 \times 10^4$). For the 1 M

Table 1
Parameters resulting from free SOE analyses of fluorescence decay profiles of DNA/Eb solutions: (a) 1M NaCl, (b) 0.05M Nacl

a) DNA:Eb	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	DW	χ^2
0.36	2.0	83.5	19.1	16.5	1.91	1.18
0.72	2.1	75.3	20.4	24.7	1.95	1.34
1.45	2.0	68.6	21.2	31.4	2.07	1.27
2.7	2.0	53.9	22.1	46.2	2.08	1.31
5.6	2.0	48.8	22.8	51.8	2.20	1.08
14.4	1.9	33.7	23.3	66.3	1.82	1.22

b) DNA:Eb	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	DW	χ^2
0.36	1.9	83.4	15.0	16.6	1.52	1.22
0.72	1.8	74.7	17.0	25.4	1.69	1.25
1.45	2.3	47.8	18.0	52.2	1.32	1.54
2.7	2.4	41.2	19.9	58.8	1.26	1.69
5.6	2.6	28.2	20.8	71.8	1.22	2.08
14.4	5.6	15.4	22.3	84.6	1.38	1.81

NaCl solutions a slightly reduced fluorescence lifetime of 1.71 ns was obtained.

Fluorescence decays of all DNA/Eb solutions were collected at both salt concentrations (0.05 M and 1 M NaCl). The results of bi-exponential SOE analyses are given in Table 1. For the 1 M salt solution, fits are of adequate statistical quality ($\chi^2 < 1.35$). For the 0.05 M salt solutions the fits are poor, notably when the DNA:Eb ratio is greater than 1.45 ($1.22 < \chi^2 < 2.08$). This suggests that the bi-exponential decay function is inadequate in describing the full decay kinetics of the DNA:Eb system. It has been proposed that as the ionic salt concentration is decreased the extent of secondary binding between Eb and DNA will increase [18,26]. Secondary binding, resulting in an additional lifetime component would account for the failure of the bi-exponential model to properly describe decays resulting from the 0.05 M NaCl solutions. Consequently, generic models of the DNA:Eb system previously proposed (at lower precision) must be questioned.

Any analysis using the SOE technique would be incomplete without reference to global analysis procedures [27]. If fluorescence decays originate from a system containing non-interacting species present in varying amounts, then the decay times will be invariant of dataset; hence *only* the magnitude of the pre-exponential factors will change. Global SOE analysis allows multiple datasets to be analysed simultaneously, where *n* lifetimes common to all datasets are reconstructed. Accordingly, global SOE analyses were performed on both set of decays (i.e. at each salt concentration). Although the global analysis is still model dependent, the application of a model over a multi-dimensional data surface is as statistically rigorous a test of a particular model as can be performed. If the model satisfies all the statistical parameters, then there is nothing more that can be done to test the particular model. Hence, global analysis is a powerful method of testing the efficacy of the SOE approach. Nevertheless, it is noted that global analysis still incorporates a priori assumptions, and is therefore still prone to curve parameterisation phenomena.

Global analysis was performed on each set of fluorescence decays, i.e. decays from the 1 M and 0.05 M salt solutions. Using a bi-exponential decay model, poor statistical fits result, (global χ^2 of 2.77 and 4.23 respectively). This suggests that a bi-exponential decay model is *not* sufficient to fully describe *all* of the decays in a given set. The complexity of the model was thus increased to incorporate a tri-exponential decay function. Now the fits

Table 2
Parameters resulting from tri-exponential global analyses of fluorescence decay profiles of DNA/Eb solutions: (a) 1M NaCl, (b) 0.05M NaCl

a) DNA:Eb	Amplitude/%			DW	χ^2
	$\tau_1 = 2.1$ ns	$\tau_2 = 18.5$ ns	$\tau_3 = 23.4$ ns		
0.36	81.07	16.38	2.55	1.89	1.17
0.72	75.32	15.75	8.93	1.80	1.37
1.45	67.89	13.90	18.21	2.00	1.31
2.8	52.78	12.82	34.40	2.11	1.24
5.6	47.89	7.93	44.19	2.33	1.10
14.4	30.85	2.04	67.11	1.90	1.24

b) DNA:Eb	$\tau_1 = 1.7$ ns	$\tau_2 = 15.1$ ns	$\tau_3 = 24.7$ ns	DW	χ^2
0.36	83.40	16.65	0.97	1.71	1.09
0.72	73.57	20.17	6.26	1.94	1.11
1.45	47.93	35.50	16.58	1.95	1.06
2.8	40.59	35.58	23.83	1.87	1.12
5.6	24.12	37.71	38.17	1.95	1.32
14.4	9.11	34.47	56.42	1.87	1.32

are of excellent statistical quality (global χ^2 of 1.29 and 1.23). The three recovered decay times for each system along with the pre-exponential factors are shown in Table 2. In addition, Figs. 3 and 4 show the decays, fits and residuals resulting from the global analyses. The random distribution of the residuals around zero and the low global χ^2 values indicate that the tri-exponential decay function is an excellent *statistical* description of both systems. The short decaytime component τ_1, describes free Eb in

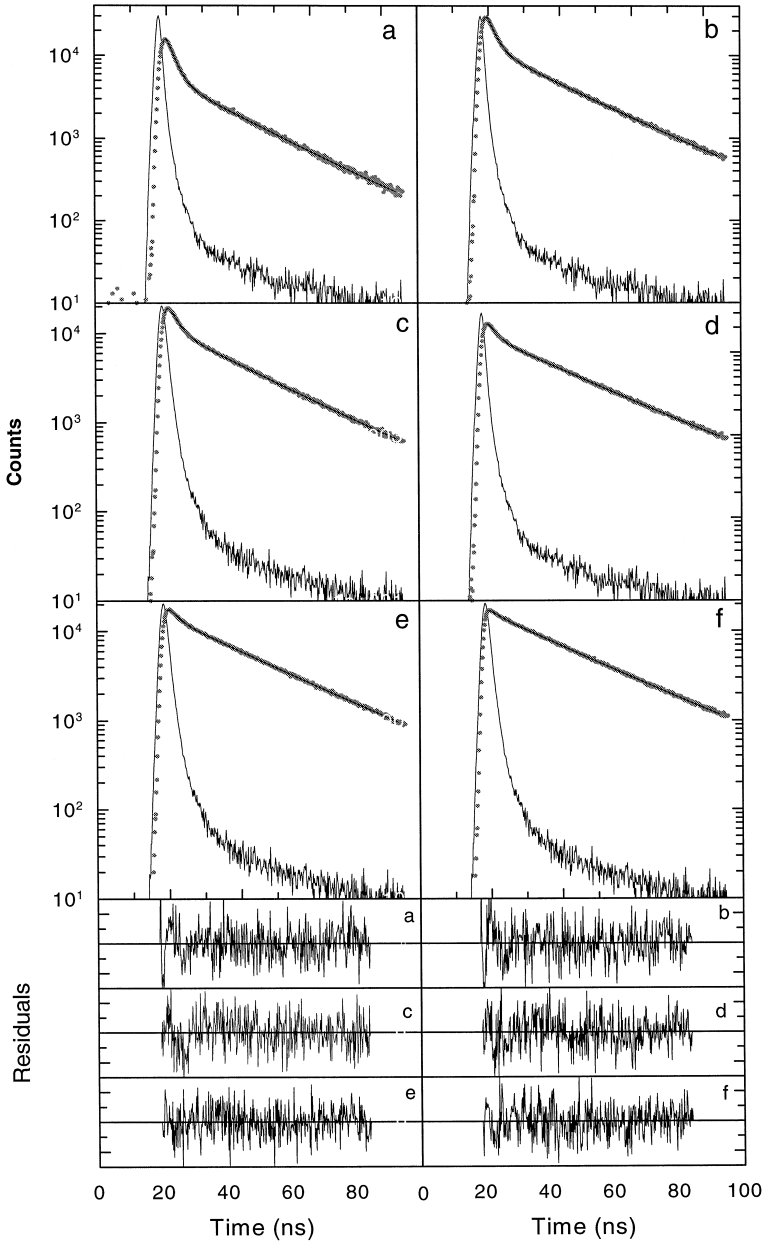

Fig. 3. Decays, IRFs and fits resulting from 3 component global analyses of the 1M NaCl, DNA/Eb solutions. DNA:Eb ratio - (a) 0.36, (b) 0.71, (c) 1.45, (d) 2.8, (e) 5.6 and (f) 14.4.

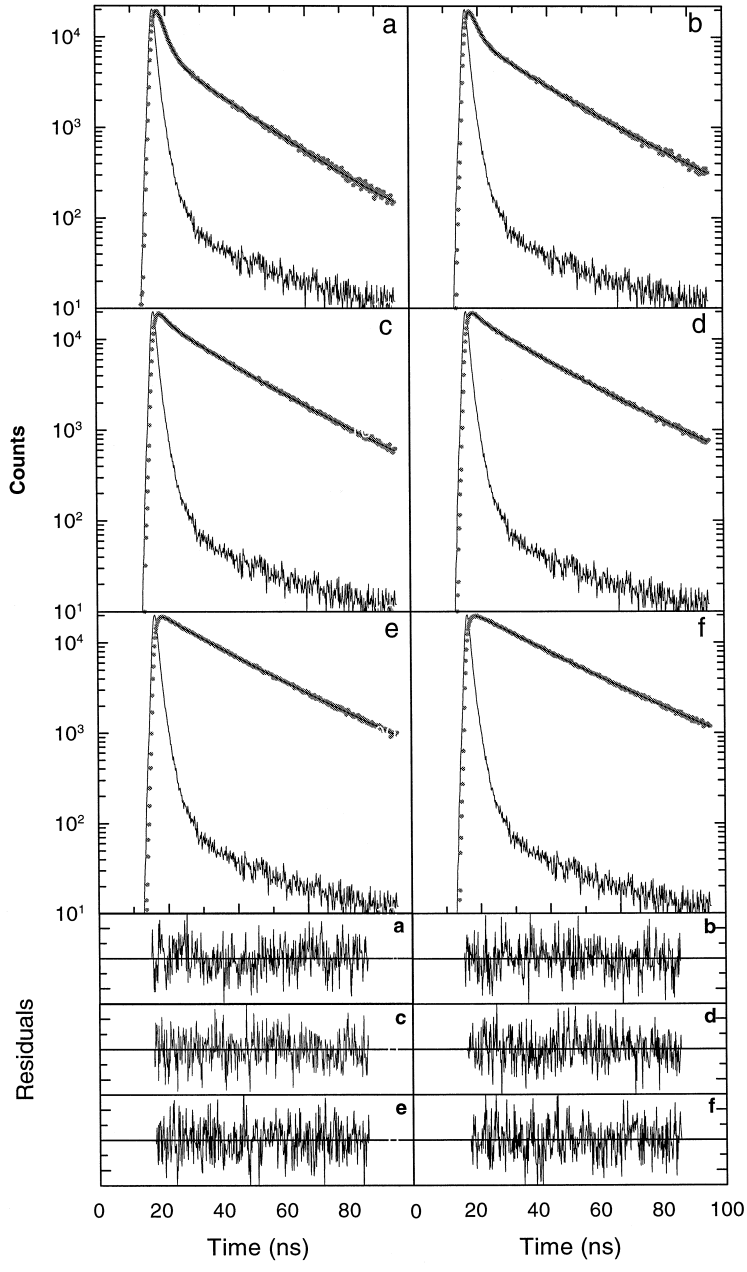

Fig. 4. Decays, IRFs and fits resulting from 3 component global analyses of the 0.05M NaCl, DNA/Eb solutions. DNA:Eb ratio - (a) 0.36, (b) 0.71, (c) 1.45, (d) 2.8, (e) 5.6 and (f) 14.4.

the system, while the longer decaytime components represent dye interacting with the ds-DNA. If the three component model of the system is correct, τ_3 describes the fully intercalated Eb and τ_2 describes Eb bound less tightly (to a secondary site). At both salt concentrations the relative amplitude of free dye is seen to increase as a function of Eb concentration, whereas the relative amplitude of the longest decay-

time component is seen to decrease as a function of Eb concentration. In addition, the variation in the relative amplitude of the intermediate decaytime component, as well as being sensitive to Eb concentration is also dependent on the overall salt concentration. For the 1 M NaCl solutions, the relative amplitude of the intermediate lifetime component increases as Eb concentration is increased. Conversely, for the 0.05 M NaCl solutions, the relative amplitude is seen to decrease as a function of Eb concentration.

At 1 M NaCl, and low [Eb] little secondary binding is expected. As Eb concentration is increased, the amount of free dye increases, resulting in distortion of the DNA structure [8]. This conformational change allows dye molecules into the previously unoccupied binding sites along the DNA strand. Consequently, the relative amplitude of the fully intercalated component decreases. As more free Eb is introduced, the DNA structure will continue to distort allowing greater accessibility of free Eb to the secondary site. Accordingly the amplitude of the intermediate decaytime component increases. For the low salt solutions (0.05 M NaCl) secondary binding is more favourable [17,18,26]. Even at low [Eb] there is appreciable secondary binding ($A_2 \sim 35\%$ in the three component analysis). As the concentration of Eb is increased the relative amplitude of the intermediate component stays approximately constant suggesting that secondary sites are saturated. As Eb concentration is increased further, continuing distortion of DNA structure will provide greater solvent accessibility to dye bound at these secondary sites. Quenching of bound states by solvent will result, and a reduction in the relative amplitudes of both the intermediate and long decaytime component are observed. This proposed model may shed light on the true physical characteristics of the DNA/Eb system. However, as was stated previously the SOE approach may lead to curve parameterisation, i.e. statistically adequate but physically meaningless information. To address this eventuality data sets were reanalysed using the MEM [22,23].

Experimentally obtained fluorescence decays were analysed using a 100 term probe function between 100 and 35 000 ps. In addition, two types of synthetic decays were generated to act as controls in the analysis. In the first, decays were simulated using parameters obtained from the *free*, *bi-exponential* fits (Table 1). In the second, decays were simulated using parameters obtained from the *global*, *tri-exponential* fits (Table 2). The resulting lifetime distributions were then compared with those resulting from analysis of the experimental data.

Figs. 5 and 6 show the comparison between real and simulated decaytime distributions for each of the DNA/Eb solutions. MEM analyses of the simulated bi-exponential decays at both salt concentrations show two discrete, narrow components in all cases. Maxima correspond to the decaytime parameters used in simulation.

MEM analyses of the simulated tri-exponential decays show a narrow component peaking at the position of the short decaytime used in simulation (i.e. 1.7 or 2.1 ns). For all datasets the MEM failed to fully resolve the two longer decaytime components τ_2 and τ_3. Instead a broad, asymmetric lifetime distribution ($0.3 < skewness < 0.5$) results, with a maximum lying between the two decaytime components used in simulation (i.e. 18.5 and 23.4 ns or 15.1 and 24.7 ns). No sharpening of the longtime distribution was possible by simulating datasets to higher precision ($c_{max} < 10^5$). Furthermore, the longtime distribution component is broader at the lower NaCl concentration (due in part to the fact that the decaytime components, used in simulation are further apart at lower salt concentrations). As the

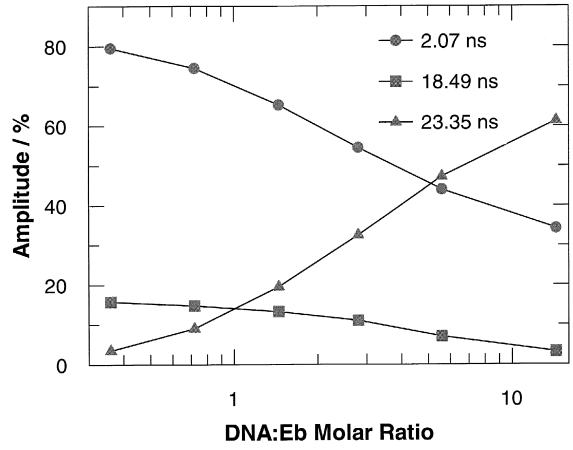

Fig. 5. Variation of component amplitudes as a function of DNA:Eb molar ratio for the 1M NaCl solutions. Parameters recovered from global, tri-exponential analyses (Table 2a).

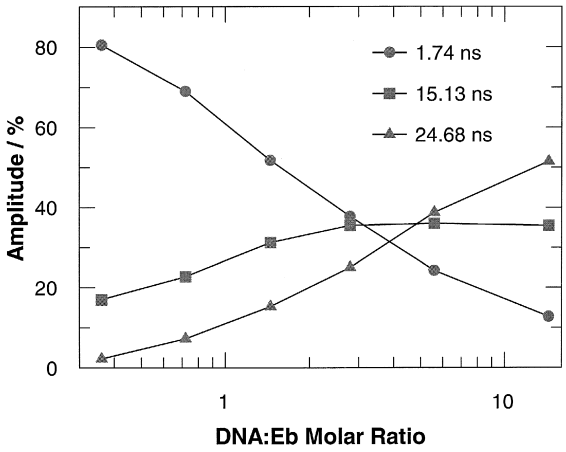

Fig. 6. Variation of component amplitudes as a function of DNA:Eb molar ratio for the 0.05M NaCl solutions. Parameters recovered from global, tri-exponential analyses (Table 2b).

DNA:Eb ratio increases, the area normalised amplitude of the long lifetime distribution increases, and shifts to longer times. This is consistent with an increase in the average fluorescence decaytime.

MEM analyses of the experimental data also failed to resolve three discrete components. Nevertheless, the lifetime distributions are almost identical to those recovered from analysis of the tri-exponential simulated datasets. For each decay, a narrow component characterising free Eb is observed along with a broader distribution at longer times. For both salt concentrations the position of this maximum is seen to increase as a function of DNA:Eb ratio. Again the longer lifetime distribution is wider at the lower (0.05 M) salt concentration. These observations provide firm evidence that a *two* component model is inadequate in describing the true kinetics of the DNA/Eb system. Furthermore, the MEM analyses suggest that a *three* component model is a far more valid description of the physical nature of the system (at this level of precision there is no clear evidence for a distributed longtime component).

6. Discussion

The dependence of the average decaytime of Eb on the DNA:Eb ratios and salt concentration was first reported by Burns [7], mentioned by Olmstead and Kearns [13] and demonstrated more recently by Atherton and Beaumont [8]. In the latter study, the variation in emission intensity was attributed to quenching by Eb bound at a secondary site. Nevertheless, as was demonstrated in Ref. [5] no intermediate lifetime component was detected. In the studies by both Atherton and Greenstock, the decaytime of intercalated Eb was seen to decrease as the DNA:Eb molar ratio decreased. In contrast, the decaytime of Eb intercalated to DNA has also been reported to be independent of DNA:Eb ratio [28]. Furthermore, it has also been proposed that free Eb in solution may distort the structure of the DNA strand. This results in greater solvent accessibility to the binding sites at higher Eb concentrations [8]. Any proposed model should be able to account for these observations.

In the work described in this paper, decaytimes and pre-exponential factors recovered from the bi-exponential SOE analyses are in agreement with those reported in [5]. However, fits are of poor statistical quality for the low salt concentration solutions ($c_{max} \sim 20\,000$). For low precision data ($> 10\,000\ c_{max}$) it has previously been shown that complex decays can be satisfactorily described by simple, exponential models [21,22]. As the precision of the data is increased, a simple model will fail to adequately describe a complex decay and fits become statistically poor (evidence that the assumed model is incorrect). In Ref. [5] decays are collected to 5000 c_{max}. At this level of precision, fluorescence decays will almost always be adequately described by simple exponential models (even if the decay is 'really' more complex). However, analyses at higher precision ($> 20\,000\ c_{max}$) demand more rigorous statistical requirements. This results in unsatisfactory fits to simple exponential models. Consequently, the applicability of kinetic models can only be rigorously tested when data is measured to high levels of precision. In particular, it has been demonstrated that a three component decay collected at low precision can be statistically described by a two component model, and that at low precision the variation in the amplitudes of the two longer decaytime components can be interpreted as a shift in the decaytime of one intermediate component. Essentially, analysis of decay data collected to a higher precision allows the resolution of all three decay components. Accordingly, it appears that a bi-exponential description of Eb decay kinetics is incorrect. Global analysis adds

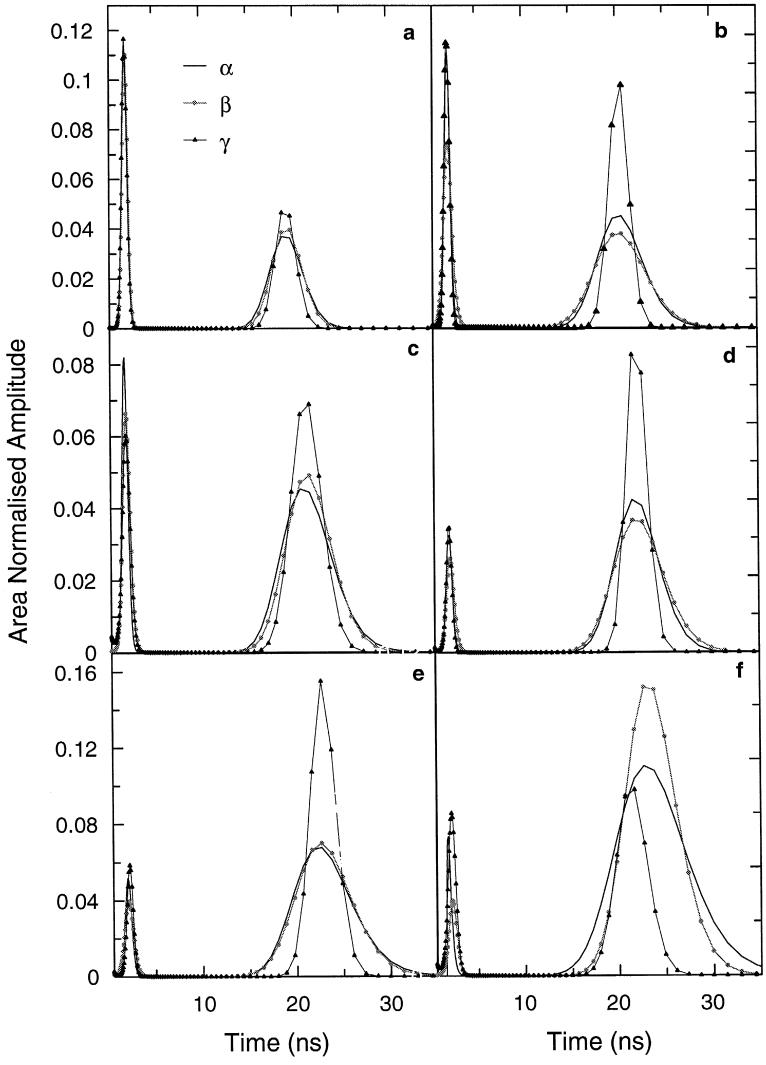

Fig. 7. MEM analyses of experimental and simulated fluorescence decays of the 1M NaCl, DNA/Eb solutions. DNA:Eb ratio - (a) 0.36, (b) 0.71, (c) 1.45, (d) 2.8, (e) 5.6 and (f) 14.4. Probe function of 100 terms over a 100-35 000 ps range: α, experimental data; β, global, tri-exponential, simulated data; γ, bi-exponential, simulated data.

weight to this hypothesis. Results indicate that a three component model more closely describes the DNA/Eb system. This model allows for the existence of a term that describes emission from Eb bound at secondary sites along the DNA backbone. The model is intuitively appealing, since it would be expected that all decays should contain a term representative of free dye, a term representative of fully intercalated dye, and a term describing emission from dye bound at secondary sites (the relative amounts of each molecular population being given by the magnitude of the pre-exponential factors). Although this model is superficially more complex than two component models proposed elsewhere, the same model can be applied to all decays at a given salt concentration, i.e.

$$I(t) = a_1 \exp(-t/1.7) + a_2 \exp(-t/15.1) + a_3 \exp(-t/24.7) \quad (5)$$

Here, decaytime components are given in ns, and as already noted, only pre-exponential factors vary as a function of [Eb]; decaytimes are fixed.

Finally, utilisation of the MEM in combination with synthetic data lends even more support to the idea of a three component system. Although the MEM cannot fully discriminate between the two long-time components of the tri-exponential model, the use of synthetic data demonstrates that experimental decays are best described by reference to a tri-exponential model (Figs. 7 and 8). Furthermore, the inherent lack of theoretical bias and the ability to recover the coefficients of an exponential series with fixed lifetimes that are free from any artifactual correlations, makes the MEM an immensely powerful tool in the analysis of fluorescence decays originating from heterogeneous media.

The studies presented in this paper are significant, since they serve to illustrate the fact that decays originating from inherently complex media can be

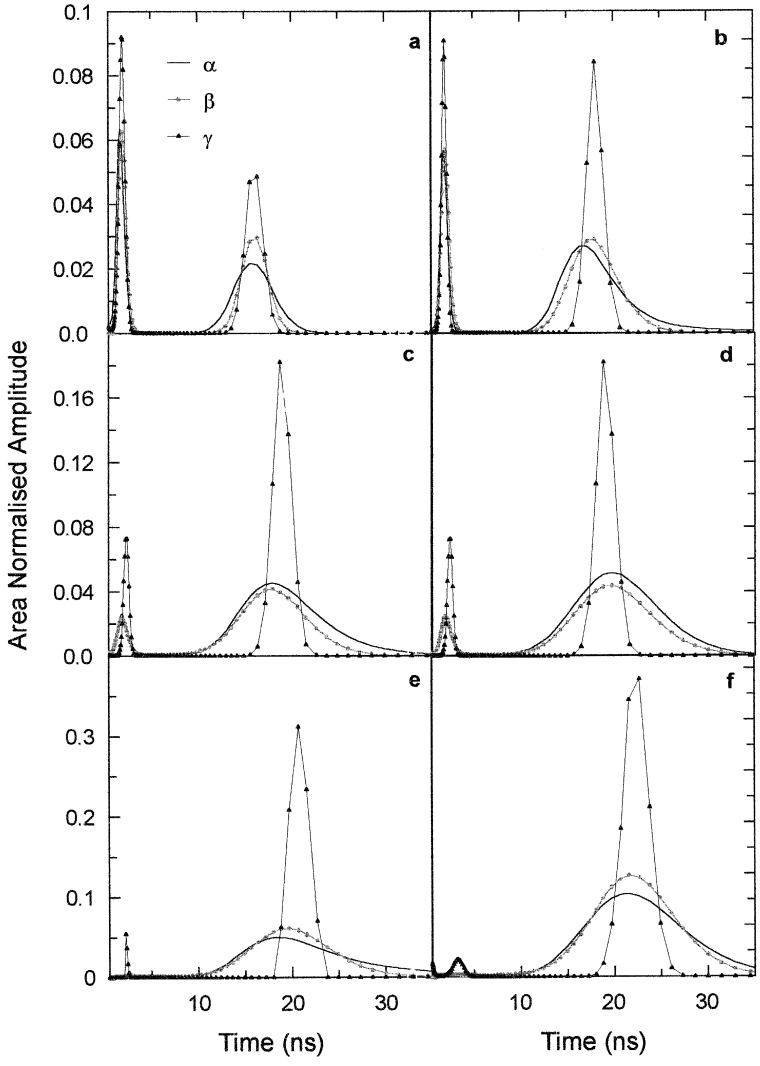

Fig. 8. MEM analyses of experimental and simulated fluorescence decays of the 0.05M NaCl, DNA/Eb solutions. DNA:Eb ratio - (a) 0.36, (b) 0.71, (c) 1.45, (d) 2.8, (e) 5.6 and (f) 14.4. Probe function of 100 terms over a 100-35 000 ps range: α, experimental data; β, global, tri-exponential, simulated data; γ, bi-exponential, simulated data.

paramaterised by relatively simple SOE models. Consequently, little physical significance should be attributed to parameters resulting from any SOE analysis *unless* supporting evidence is available. In this case, MEM analyses and the use of synthetic data provide strong evidence for a three component model, where each component is physically relevant.

7. Conclusion

In conclusion, as Greenstock says, caution must be taken when using in vitro or biochemical assays that infer a change in the amount of ds-DNA through variations in the time-integrated fluorescence intensity. Current studies are addressing the precise nature of the secondary binding sites along the DNA backbone.

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