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Associated Anisotropy Decays of Ethidium Bromide Interacting with DNA

Rahul Chib^{1,*}, Sangram Raut¹, Sarika Sabnis¹, Preeti Singhal¹, Zygmunt Gryczynski^{1,2}, and Ignacy Gryczynski^{1,*}

¹Department of Cell Biology and Immunology, Center for Fluorescence Technologies and Nanomedicine, University of North Texas Health Science Center, Fort Worth, TX 76107, USA

²Department of Physics and Astronomy, Texas Christian University, Fort Worth, TX, 76129, USA

Abstract

Ethidium Bromide (EB) is a commonly used dye in a deoxyribonucleic acid (DNA) study. Upon an intercalation, this dye significantly increases its brightness and fluorescence lifetime. In this report we have studied the time-resolved fluorescence properties of EB existing simultaneously in free and DNA-bound forms in the solution. Fluorescence intensity decays were fitted globally to a double exponential model with lifetimes corresponding to free (1.6ns) and bound (22ns) forms, and molar fractions were determined for all used solutions. Anisotropy decays displayed characteristic time dependence with an initial rapid decline followed by recovery and slow decay. The short-lived fraction associated with free EB molecules decreases faster than long-lived fraction associated with EB bound to DNA. Consequently, contribution from fast rotation leads to initial rapid decay in anisotropy. On the other hand bound fraction, due to slow rotation helps recover anisotropy in time. This effect of associated anisotropy decays in systems such as EB free/EB-DNA is clearly visible in a wide range of concentrations, and should be taken into account in polarization assays and biomolecule dynamics studies.

Keywords

Associated anisotropy decays; Ethidium Bromide and DNA fluorescence

Introduction

Time-resolved anisotropy of free fluorophores in solutions usually shows an exponential decay with a single correlation time. Multiple correlation times are sometimes observed for single fluorophores displaying complex motions [1]. A more complex situation arises when a fluorophore is bound to a macromolecule because the local motion of the dye is superimposed with an overall rotation of the macromolecule. In general, time-resolved anisotropy of bound fluorophores yield important information about the size, shape and different dynamic properties of biological molecules. When the dye is bound to a biomolecule, its photophysical properties tend to change due to restricted mobility and a

*Corresponding Authors: Rahul Chib (Rahul.chib@live.unthsc.edu), Ignacy Gryczynski (Ignacy.gryczynski@unthsc.edu).

change in its microenvironment. Often, the fluorescence efficiency, lifetime and spectra of a fluorophore show substantial changes with such binding. The observed fluorescence properties are sometimes complex if the system under investigation has free and bound forms of fluorophores. In the case of associated anisotropy decay, the same fluorophore is present in more than one type of microenvironment; because of this, it behaves differently and has different excited state lifetimes leading to different depolarization processes in time. Early studies of associated decay was started using a probe in which cis-parinaric acid was covalently linked to phosphatidylcholine molecule and an increase/recovery in anisotropy at times longer than 10 ns was observed [2]. Associated decay of labeled oligonucleotides bound to Klenow fragment was also observed [3]. This type of anisotropy decays are mostly multi-exponential due to the presence of multiple emitting species, each of which displays its own anisotropy decay [4].

The interaction between DNA and EB is one such system, which shows a metachromatic effect, and from which a lot of quantitative data can be obtained [5]. When EB intercalates between the nucleotides of DNA, its photophysical properties change drastically. With increasing concentration of bound EB, the fluorescence lifetime and anisotropy increases until equilibrium is achieved which correspond to one EB molecule for every 5 nucleotides [6,7]. The fluorescence intensity of free EB is quenched by molecular oxygen in the aqueous environment and therefore unbound dye is only weakly fluorescent [8]. The intercalation of EB molecules in hydrophobic environment of DNA provides a steric protection and prevents the quenching by solvated oxygen. The fluorescent quantum yield of the EB increases considerably on binding to the nucleic acid [9].

Various experiments have been done by numerous scientists to study the interaction of chromatin material with different fluorophores to determine the physical properties and dynamics of chromatin material. Heller [8] suggested in their paper that an increasing concentration of unbound EB in system containing EB and DNA will shorten the fluorescence lifetime and the fluorescence intensity of bound EB. Therefore the use of steady state fluorescence intensity to estimate the concentration of DNA must be interpreted with caution. Genest et al.[10] studied the anisotropy decay of ethidium bromide bound to nucleosome core material and suggested that the first bound EB molecule is located on a DNA segment linked by its two ends to histone core and this EB follows the torsional motion of the DNA. Anisotropy decay of EB intercalated in DNA was studied by Barkley and Zimm [11], Allison and Schurr [12] and Thomas et al [13]. It has been suggested that the depolarization of fluorescence is due to the torsional motion of DNA around its axis. Another paper by Genest et al. [14] shows the fluorescence anisotropy decay of the chromatin complex and reported that high affinity sites for binding are clustered on a short nucleosomal DNA fragment. Waring et al. [15] in their paper concluded that the complex formation between EB molecule and nucleic acid is not influenced by base composition of DNA but is sensitive to changes in salt concentration, particularly in the presence of magnesium ion. However, all of the above studies did not consider the associated anisotropy decays of free and bound EB.

In this study, we demonstrate the importance of associated anisotropy decays in experiments involving DNA and EB. If a system consists of free and bound EB, both of these emitting

species will contribute towards total anisotropy. More importantly if species have significantly different fluorescence lifetime the relative fractions contributing to the overall anisotropy decay will be quickly changing during the decay. Not considering the involvement of both of the emitting species can lead to an error in quantification or interpreting dynamic properties of DNA and without this consideration, faulty assumptions can be made. The measurements of EB-DNA associated anisotropy decays were supported by simulations, which used the associated anisotropy concept with different ratios of bound and unbound fractions. With this type of simulation one can determine the anisotropy decay pattern of any associated system.

Material and methods

Ethidium bromide and DNA sodium salt from calf thymus were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). 1X phosphate buffer saline (PBS) of pH 7.4 was purchased from Invitrogen, Life Technologies (Invitrogen Corporation, CA, and USA).

Samples preparation

Two solutions were prepared, first, 36.9 μM of EB in PBS (solution A), and a mixture of 36.9 μM of EB and 585 μM of DNA (solution B). Absorption, fluorescence spectra, steady state and time resolved anisotropy along with fluorescence lifetime of these two solutions were measured. Next, solution B in increasing volume was added to the solution A which keeps the molar concentration of EB constant whereas the molar concentration of DNA was progressively increased. Fluorescence lifetime and time resolved anisotropy decay of these solutions were measured.

Steady state fluorescence measurements

Steady state fluorescence intensity of all the solutions was measured using Carry Eclipse spectrofluorometer (Varian Inc., Australia) using 485 nm excitation and a 495 nm long pass filter along emission side. Steady state anisotropy of two solutions (solution A and solution B) was measured using Carry Eclipse spectrofluorometer (Varian Inc., Australia) with manual polarizers on both excitation and emission side. Emission anisotropy was measured using 485 nm excitation light and 495 nm long pass filter along emission side and manually operated parallel and perpendicular polarizers. For steady state excitation anisotropy, emission was observed at 605 nm and excitation was scanned from 400 nm to 580 nm using a 590 nm long pass filter on the emission side. Anisotropy was calculated using formula:

$$r = \frac{I_{VV} - I_{VH}G}{I_{VV} + 2I_{VH}G} \quad (1)$$

Where, I_{VV} is the fluorescence intensity measured with the parallel polarizer orientation on the observation path, I_{VH} is the fluorescence intensity at the perpendicular orientation of the polarizer and G is the instrumental correction factor.

Fluorescence lifetimes and time resolved anisotropy measurements

Fluorescence lifetime and anisotropy decays of all the solutions were measured using FluoTime 200 (PicoQuant, GmbH, Berlin, Germany) time domain fluorometer. This instrument contains multichannel plate detector (Hamamatsu, Japan) and a 485 nm laser diode was used as the excitation source. The fluorescence lifetime was measured in magic angle conditions and data was analyzed with FluoFit version 5.0 software (PicoQuant GmbH, Berlin, Germany) using a multiexponential deconvolution model:

$$I(t) = \int_{-\infty}^t IRF(t') \sum_i \alpha_i e^{-\frac{t-t'}{\tau_i}} dt' \quad (2)$$

Where IRF (t) is the instrument response function at time t', α_i is the amplitude of the decay of the i-th component at time t and τ_i is the lifetime of the i-th component.

Excitation used for time resolved anisotropy measurement was 485 nm while emission was observed at 625 nm with vertical and horizontal polarizer position on the emission side using appropriate filters on both excitation and emission side. Anisotropy decay was analyzed with an associated anisotropy decay model using fitting routine design in MathCad 15 platform.

Associated anisotropy decay analysis and simulations

The system like EB-DNA is typically in a stable equilibrium (within the fluorescence lifetime) between the fraction of unbound fluorophores and bound fluorophores and time-dependent emission anisotropy is described by contributions of both fractions and their respective initial anisotropies r_1 and r_2 :

$$r(t) = f_1(t) \cdot r_1 e^{-t/\Phi_1} + f_2(t) \cdot r_2 e^{-t/\Phi_2} \quad (3)$$

Where f_1 represent an initial fraction of unbound EB, τ_1 - fluorescence lifetime of unbound EB, Φ_1 correlation time of unbound EB, f_2 - initial fraction of bound EB, τ_2 - fluorescence lifetime of bound EB, Φ_2 correlation time of bound EB and for any given moment of time, $f_1(t) + f_2(t) = 1$ represent normalized fractions. The fractions at any time t are given by:

$$f_1(t) = \frac{\alpha_1 \exp(-t/\tau_1)}{\sum \alpha_i \exp(-t/\tau_i)} \quad (4)$$

$$f_2(t) = \frac{\alpha_2 \exp(-t/\tau_2)}{\sum \alpha_i \exp(-t/\tau_i)} \quad (5)$$

Where α_1 and α_2 are fractional amplitudes of unbound and bound fractions, respectively.

The equation (3) shows that measured anisotropy at initial time depends on initial fractions and their initial anisotropies. Both fractions evolve accordingly to their respective

fluorescence lifetimes and their relative contribution is constantly changing. Also the respective anisotropies for each fraction changes accordingly to their correlation times.

Rotational diffusion of unbound fluorophores (free EB) is fast as well as represented by single correlation time Φ_1 . Time dependent anisotropy decay can be described as:

$$r_1(t) = r_{01} e^{-t/\Phi_1} \quad (6)$$

As EB binds to the DNA its rotational freedom is much smaller. Depending on the place where EB has been bound it can move due to binding site internal motion, due to the segmental and torsional DNA motions. Its motion will be represented with a complex correlation time given by:

$$r_2(t) = \sum_i r_{02}^i e^{-t/\Phi_{2i}} \quad (7)$$

Where, i represents different fractions of bound EB in DNA.

It is important to stress that observed initial fractions will depend directly on the equilibrium (bound and unbound) as well as on the extinction coefficient at the excitation wavelength and quantum yield of bound and unbound fluorophores. In general, the extinction coefficient and quantum yield can be different for both forms. It is interesting to consider a few examples. For simplicity we will assume that extinction coefficients and quantum yields at the excitation wavelength as well as initial anisotropies are identical for both fractions. If they are different for both forms a simple correction factor can be calculated.

Results

Steady-State fluorescence

As shown in Figure 1, with increase in concentration of bound EB, the fluorescence emission intensity also increases. The free, unbound EB has low fluorescence quantum efficiency (0.023) calculated using EB in methanol as reference [16]. Quantum efficiency quickly increases with increase in bound fraction and attaining highest value of 0.40 in saturated DNA. The intercalation of EB molecules inside DNA nucleotides results in higher brightness.

Fluorescence lifetimes

Fluorescence lifetime of EB also increases after binding with DNA which is shown in Figure 2. Fluorescence lifetime of free EB in PBS is 1.6 ns whereas after binding with DNA it increased to 22.05 ns. This increase in lifetime can be attributed to hydrophobic microenvironment which protects its interaction with water molecules and molecular oxygen. The intensity decays of all samples were analyzed with global lifetimes, 1.6 ns for unbound EB and 22.05 ns for EB-DNA. Fractional amplitudes of EB with different concentration of DNA are given in Table 1. The fractional amplitude of the bound fraction increases with increased DNA concentration as it provides more nucleotides to bind to. This

increase of the bound fraction of EB with the addition of DNA can be easily observed in the representative fluorescence intensity decays (Figure 3).

Fluorescence anisotropy

Steady state excitation and emission anisotropy of both, free and bound EB are shown in Figure 4. After binding to DNA, EB shows blue shift (~ 20 nm) in the emission spectrum. Anisotropy of free EB is close to zero due to very fast rotation of EB molecules in water. Steady state anisotropy of EB bound to DNA is 0.17, significantly higher than for free form. This increase in anisotropy is due to the intercalation of EB molecules inside DNA which results in the immobilization of EB molecules. A depolarization of EB-DNA fluorescence depends on slow torsional DNA motions. In effect, the anisotropy decay of EB-DNA is complex and shows longer rotational correlation times.

We analyzed anisotropy decays using the associated decay model (eq. 3). The time dependent fractions of unbound EB and EB-DNA were calculated from the intensity decays (eq. 4,5 Table 1)

Time resolved anisotropy decays of free EB and bound to DNA are shown in Figure 5. For free EB only one component with correlation time of 0.15 ns is needed to fit the data. For bound EB three components with correlation time of 103.3 ns, 12.37 ns and 0.92 ns are needed to fit the data. With increasing concentration of DNA, different types of anisotropy decay were observed (Figure 6). This type of decay pattern is characteristic for associated anisotropy decays. The reason for this type of decay pattern is presence of EB fluorophores in two different forms, free and bound to DNA. After the excitation, the fluorescence anisotropy quickly decreases because the fraction of free EB decays faster. In time, the bound fraction (EB-DNA) increases and the anisotropy recover; and finally slowly decay because only EB-DNA form remains in the excited state. One cannot rule out or ignore the anisotropy contribution of fast decaying component and recovery due to bond fraction while studying dynamics of biomolecule such as DNA.

Simulations of associated anisotropy decays

Using an associated anisotropy concept, we simulated the anisotropy decays using parameters corresponding to free EB and EB-DNA mixtures. We assumed two emitting species with lifetimes of 2 ns and 20 ns, and corresponding correlation times 2 ns and 50 ns. The initial anisotropy was assumed 0.4. We simulated the data for different ratios (different fractional amplitudes) of these two species. These simulations are shown in figure 7. From the figure 7, one can observe the rapid decrease of the observed anisotropy followed by a slower increase and that a slow decay; changes consistent with time-dependent changes of the fractions of emitting species. Profiles of simulated anisotropy decays (Figure 7) mimic these measured for mixtures of free and bound EB (Figure 6).

Conclusions

Associated anisotropy decay is a special type of anisotropy decay pattern, observed in a system in which fluorophore is present in more than one form characterized with different lifetimes and rotational correlation times. The fraction of fluorophore with short lifetime and

short correlation time (usually free fluorophores) will decrease rapidly after the excitation while the fraction of long lifetime and long correlation time (usually bound fluorophores to macromolecule) will increase. Therefore, in the measured anisotropy decays one can observe initial drop and increase of anisotropy values followed by a slow decay characteristic for a bound form. Exactly this happened for the mixtures of EB free form and EB-DNA form. We believe that assays involving EB and DNA should be analyzed with the associated decay model. Neglecting this type of decay pattern can lead to false interpretation of results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Fluorescence emission spectra of ethidium bromide with different molar concentrations of DNA.

Figure 2.

Fluorescence intensity decays of free EB and saturated EB-DNA using 485 nm laser diode for the excitation. Fluorescence lifetime of free ethidium bromide is 1.6 ns and that of EB-DNA is 22.06 ns. Decays were fitted using multi-exponential model and chi-square values were used to access goodness of fit.

Figure 3.

Fluorescence intensity decays of EB samples with increasing molar concentration of DNA. Fractional amplitude of bound EB component increases with DNA concentration. (Omitted decays are in supporting information, Figure S1)

Figure 4.

Excitation and emission spectra along with respective excitation and emission anisotropies of free and bound EB. Excitation wavelength of 485 nm was used for emission spectra and anisotropy. For excitation spectra emission was observed at 605 nm

Figure 5.

Fluorescence anisotropy decays of free EB and EB-DNA. The anisotropy decay of free EB can be approximated with a single correlation time of 0.15 ns. The anisotropy decay of EB-DNA requires three correlation times of 103.3 ns, 12.37 ns and 0.92 ns for a satisfactory fit.

Figure 6.

Associated anisotropy decays of free EB and EB-DNA mixtures; with EB- 36.9 μM and DNA-0 μM (Green), DNA- 5.85 μM (Red), DNA- 33.16 μM (Blue), DNA- 97.53 μM (Pink), DNA- 585 μM (Brown). (Omitted decays in supporting information, Figure S2).

Figure 7.

Simulation of associated anisotropy decays for two emitting species: first with a lifetime of 2 ns and correlation time of 0.2 ns; and second with lifetime of 20 ns and correlation time of 50 ns. The fractional amplitudes are: (a) 0.9/0.1, (b) 0.8/0.2, (c) 0.7/0.3, (d) 0.6/0.4, (e) 0.5/0.5, (f) 0.4/0.6, (g) 0.3/0.7, (h) 0.2/0.8, (i) 0.1/0.9.

Table 1

Analysis of EB-DNA fluorescence intensity decays with multi-exponential model.

Concentrations		Lifetime (ns)			Amplitudes			Average lifetime (ns)			Chi square
DNA (μM)	EB (μM)	τ ₁	τ ₂		α ₁	α ₂		τ _{AMP}	τ _{NT}		χ ² _R
0	36.9	1.6	-	1	1	-		1.6	1.6		1.10
5.85	36.9	1.6	22.05	0.97	0.03			2.06	6.50		1.15
17.09	36.9	1.6	22.05	0.92	0.08			3.06	12.08		1.31
33.16	36.9	1.6	22.05	0.85	0.15			4.62	16		1.33
53.22	36.9	1.6	22.05	0.74	0.26			6.96	18.57		1.26
97.53	36.9	1.6	22.05	0.46	0.54			12.59	20.84		1.20
585	36.9	-	22.05	-	1			22.05	22.05		1.10

$$\tau_{AMP} = \sum_i \alpha_i \tau_i$$

$$\tau_{INT} = \sum_i f_i \tau_i$$

Where,

$$f_i = \frac{\alpha_i \tau_i}{\sum_i \alpha_i \tau_i}$$

Table 2

Analysis of EB-DNA fluorescence anisotropy decays with associated anisotropy model.

Concentration (μM)		Anisotropy				Correlation time				Amplitudes	
DNA	Ethidium Bromide	r_{01}	r_{02}^1	r_{02}^2	r_{02}^3	Φ_1 (ns)	Φ_{21} (ns)	Φ_{22} (ns)	Φ_{23} (ns)	α_1	α_2
0	36.9	0.3	-	-	-	0.15	-	-	-	1	0
5.85	36.9	0.34	0.105	0.147	0.057	0.15	0.92	12.37	103.3	0.97	0.03
17.09	36.9	0.34	0.126	0.132	0.052	0.15	0.92	12.37	103.3	0.93	0.07
33.16	36.9	0.34	0.129	0.114	0.067	0.15	0.92	12.37	103.3	0.85	0.15
53.22	36.9	0.35	0.125	0.121	0.064	0.15	0.92	12.37	103.3	0.74	0.26
97.53	36.9	0.40	0.108	0.124	0.077	0.15	0.92	12.37	103.3	0.46	0.54
585	36.9	-	0.031	0.135	0.144	0.15	0.92	12.37	103.3	0	1