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Simultaneous Binding of Minor Groove Binder and Intercalator to Dodecamer DNA: Importance of Relative Orientation of Donor and Acceptor in FRET

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In the present study, steady-state, picosecond time-resolved fluorescence and polarization gated anisotropy have been used to establish simultaneous binding of an intercalator (ethidium bromide, EtBr) and a minor groove binder (Hoeschst 33258, H258) to a dodecamer DNA of specific sequence. The Förster resonance energy transfer (FRET) studies between the dyes H258 (donor) and EtBr (acceptor) in the dodecamer, where the ligands have a particular relative orientation of the transition dipoles, in contrast to the cases in sodium dodecyl sulfate (SDS) micelles and larger genomic DNA, where the orientations are random, reveal the effect of the binding geometry of the ligands in the constrained environment. Our study establishes that reconsideration of the value of the orientation factor (κ^2) is crucial for correct estimation of the donor−acceptor distance when the ligands are simultaneously bound to a specific region of biological macromolecules.

Introduction

The recognition of DNA by small molecules[1] is of special importance in the design of new drugs. There are several applications of these DNA-bound molecules. Some of the molecules like Hoechst 33258 (H258), 4′,6-diamidino-2-phenylindole (DAPI), and ethidium bromide (EtBr) are used as fluorescent cytological stains of DNA.[2,3] H258 is also used as a potential antihelmenthic drug.[4] Other molecules like daunomycin, netropsin, pentamidine, and berenil are well-known anticancer drugs. On the other side, many of these small ligands including EtBr bound to DNA also act as potential mutagens[5,6] (see also MSDS Sigma-Aldrich). There are three specific modes of interaction of small ligands with DNA, namely, intercalation, minor groove binding, and major groove binding. The binding of these ligands to DNA is highly sequence specific. A number of techniques like X-ray crystallography,[7] NMR,[8,9] and Raman spectroscopy[10] along with theoretical calculations[11] have been used to characterize a variety of these ligands in their specific DNA environments. However, simultaneous binding of the various ligands to DNA is an area that has not yet been sufficiently explored. Simultaneous binding of different ligands to DNA has importance in drug designing, since it provides information on the compatibility of various drugs and the effect of drugs on a mutagen-bound DNA. Since the majority of the mutagens and anticancer/antihelmenthic drugs are intercalators and minor groove binders, respectively,[4−6] the simultaneous binding of an intercalator and minor groove binder to DNA is worth investigation. The nature of the binding of an intercalator and minor groove binder to genomic DNA and mammalian cells has been reported[12,13] in the literature. In a recent report,[12] Förster resonance energy transfer (FRET) has been used to study the distance between the bound ligand molecules in genomic DNA. The study[12] shows that the region where intercalation takes place is not suited for minor groove binding in the genomic DNA. It has to be noted that, under normal conditions, both of the ligands do not compete for a particular site. The condition where both of the ligands are forced to occupy the same sites is hard to achieve in a genomic DNA solution with the very high concentration of ligands because the aforesaid condition triggers DNA condensation.[14,15] An alternative route to studying simultaneous binding of ligands is to use a smaller DNA as the host for both of the ligands. A similar case has been reported for major and minor groove binding[16] of the two ligands ruthenium−porphyrin and DAPI in a dodecamer DNA. The above-mentioned study has established resonance energy transfer across the DNA stem. To study the complexation of a minor groove binding drug on a DNA with a potent mutagenic intercalator is the motive of this work.

In our study, EtBr and H258 have been used as the model intercalator and minor groove binder, respectively. The dye EtBr is well characterized as an intercalator in different studies.[17−20] The dye shows an increase in fluorescence intensity (11 times) and lifetime (1.5 ns in bulk buffer to 22 ns) when bound to genomic as well as synthesized DNA.[17,21] The binding constant of ethidium to DNA at higher [DNA]/[dye] ratio is 10×10^5 M^{-1}.[21] Similarly, the dye H258 has been well characterized as a minor groove binder.[22−24] X-ray crystallographic and NMR studies[7,25] of the dye bound to dodecamer DNA show that the dye is bound to an A−T rich sequence of the DNA minor groove. The binding constant of the dye to double stranded DNA at low [dye]/[DNA] ratio is found to be 5×10^5 M^{-1}.[26] Solvation studies on the DNA-bound dye with femtosecond[27] and picosecond[28] resolution have identified the dynamics of biological water and relaxation dynamics of DNA environments, which are consistent with other studies.[29,30] In the present

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communication, steady-state, picosecond time-resolved emission spectroscopy and polarization gated anisotropy have been used to characterize the simultaneous binding of the ligands to the dodecamer DNA. FRET studies have been employed to estimate the distance between the two ligands. The study also establishes that the relative orientation of the transition dipoles of the donor (H258) and acceptor (EtBr) plays an important role in the energy transfer when the two ligands are simultaneously bound in a confined geometry of a dodecamer DNA.

Materials and Methods

Salmon sperm DNA and phosphate buffer are from Sigma. The dodecamer DNA, with the sequence CGCAAATTTGCG and obtained from GeneLink (USA), has been purified by the reverse phase cartridge (RPC) technique and checked by gel electrophoresis. The gel electrophoresis result indicated a single spot consistent with pure DNA. The fluorescent dyes Hoechst 33258 (H258) and ethidium bromide (EtBr) are obtained from Molecular Probes, and sodium dodecyl sulfate (SDS) is from Fluka. All of the solutions are prepared in 50 mM phosphate buffer using water from the Millipore system. The probe−DNA solutions are prepared by adding a requisite amount of the probe in DNA solution and stirring for 1 h. The circular dichroism (CD) spectra are carried out in a quartz cell having a path length of 1 cm.

Steady-state absorption and emission are measured with a Shimadzu model UV-2450 spectrophotometer and a Jobin Yvon model Fluoromax-3 fluorimeter, respectively. CD experiments are done in a Jasco 815 spectrophotometer. Fluorescence transients are measured by using a spectrophotometer from Edinburgh Instrument (LifeSpec-ps), U.K. (excitation wavelengths 375 nm, instrument response function 80 ps). The observed fluorescence transients are fitted by using a nonlinear least-squares fitting procedure to a function ($X(t) = \int_0^t E(t')\, R(t - t')\, dt'$) comprised of convolution of the IRF ($E(t)$) with a sum of exponentials ($R(t) = A + \sum_{i=1}^{N} B_i e^{-t/\tau_i}$) with pre-exponential factors (B_i), characteristic lifetimes (τ_i), and a background (A). The relative concentration in a multiexponential decay is finally expressed as $c_n = (B_n/\sum_{i=1}^{N} B_i) \times 100$. The quality of the curve fitting is evaluated by reduced chi-square and residual data. In order to estimate the Förster resonance energy transfer efficiency of the donor (H258) to the acceptor (EtBr) and hence to determine distances of donor−acceptor pairs, we have followed the methodology described in chapter 13 of ref 31. The Förster distance (R_0) is given by

$$R_0 = 0.211[\kappa^2 n^{-4} Q_D J(\lambda)]^{1/6} \quad (\text{in Å}) \tag{1}$$

where κ^2 is a factor describing the relative orientation in space of the transition dipoles of the donor and acceptor. The value of the orientation factor (κ^2) is calculated from the equation

$$\kappa^2 = (\cos\theta_T - 3\cos\theta_D \cos\theta_A) \tag{2}$$

where θ_T is the angle between the emission transition dipole of the donor and absorption transition dipole of the acceptor and θ_D and θ_A are the angles between these dipoles and the vector joining the donor and acceptor.[31] The refractive index (n) of the medium is assumed to be 1.5. Q_D, the quantum yield of the donor in the absence of acceptor is measured to be 0.54, 0.53, and 0.53 in SDS micelles, genomic DNA, and dodecamer DNA, respectively. $J(\lambda)$, the overlap integral, which expresses the degree of spectral overlap between the donor emission and the acceptor absorption, is given by

$$J(\lambda) = \frac{\int_0^\infty F_D(\lambda)\, \epsilon(\lambda)\, \lambda^4\, d\lambda}{\int_0^\infty F_D(\lambda)\, d\lambda} \tag{3}$$

where $F_D(\lambda)$ is the fluorescence intensity of the donor in the wavelength range of λ to $\lambda + d\lambda$ and is dimensionless. $\epsilon(\lambda)$ is the extinction coefficient (in M^{-1} cm^{-1}) of the acceptor at λ. If λ is in nm, then $J(\lambda)$ is in units of M^{-1} cm^{-1} nm^4. Once the value of R_0 is known, the donor−acceptor distance (r) can easily be calculated using the formula

$$r^6 = [R_0^6(1 - E)]/E \tag{4}$$

Here, E is the efficiency of energy transfer. The efficiency (E) is calculated from the lifetimes of the donor in the absence and presence of acceptors (τ_D and τ_{DA}).

$$E = 1 - (\tau_{DA}/\tau_D) \tag{5}$$

For anisotropy ($r(t)$) measurements, emission polarization is adjusted to be parallel or perpendicular to that of the excitation and anisotropy is defined as

$$r(t) = \frac{[I_{para} - GI_{perp}]}{[I_{para} + 2GI_{perp}]} \tag{6}$$

G, the grating factor, is determined following the long-time tail matching technique[32] to be 1.1. The integrity of the time constants of $r(t)$ decays is further checked by the methodology described in ref 33 and found to have good agreement with the former method.

Results and Discussion

The dye H258 is extremely sensitive to the polarity of the environment. The absorption spectrum of the dye shows a red shift, and the emission spectrum shows a blue shift with the decrease in the polarity of the environment. The strong dependence of the emission spectrum of the dye on the polarity of the environment has been exploited to characterize the polarity of the minor groove of the DNA.[23] The emission spectrum (Figure 1a) clearly shows that the dye resides in the hydrophobic environment of the SDS micelle and that of the DNAs compared to that in bulk buffer. Figure 1b shows the fluorescence transients of the probe in different environments. The temporal fluorescence decay of the probe in buffer at pH 7.0 is characterized by time constants of 110 ps (14%), 480 ps (13%), and 2.24 ns (72%). Due to the geometrical restriction imposed on the probe in the SDS micelles, the 480 ps component indicative of the twisting motion of the probe in the bulk environment[28] is lost and the fluorescence decays with time constants of 1.156 ns (14.60%) and 4.102 ns (85.40%). The geometrical restriction imposed on the dye bound to the minor groove of genomic and synthesized dodecamer is also evident from the absence of the 480 ps component in the fluorescence decays of the dye in the respective media. The probe bound to the genomic and dodecamer DNA shows only a nanosecond component in the temporal decay of fluorescence. The geometrical restriction characterizing the binding of the probe in the micellar and DNA environment is also borne out by the decay of fluorescence anisotropy in the different media, as shown in Figure 2.

Having thus characterized the binding characteristics of H258 in different environments, we consider the possibility of the simultaneous binding of H258 and EtBr in DNA. FRET is an

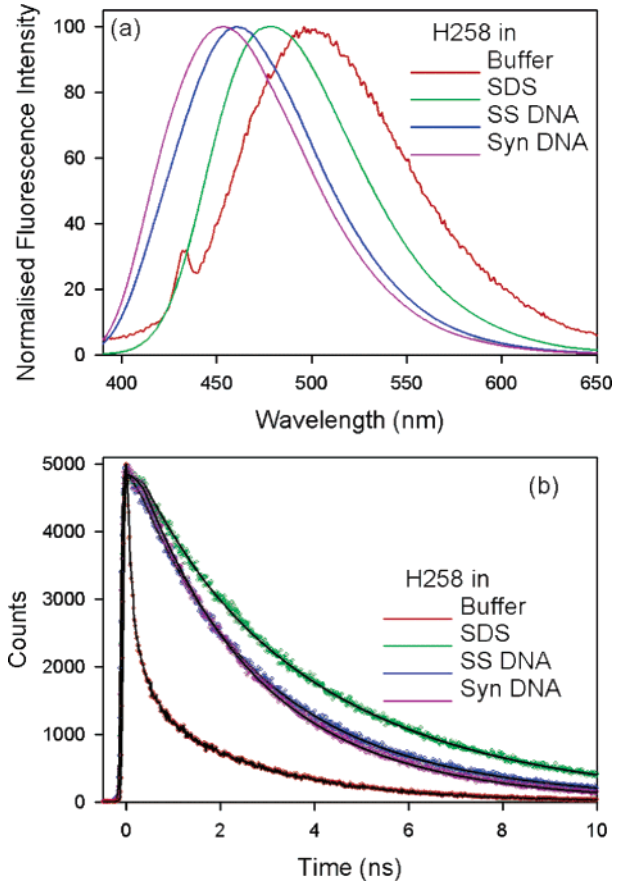

Figure 1. Emission (a) and temporal decay (b) of 1 μM H33258 (H258) in 50 mM phosphate buffer, 50 mM SDS, 100 μM (base pair) SS DNA, and 70 μM (base pair) dodecamer.

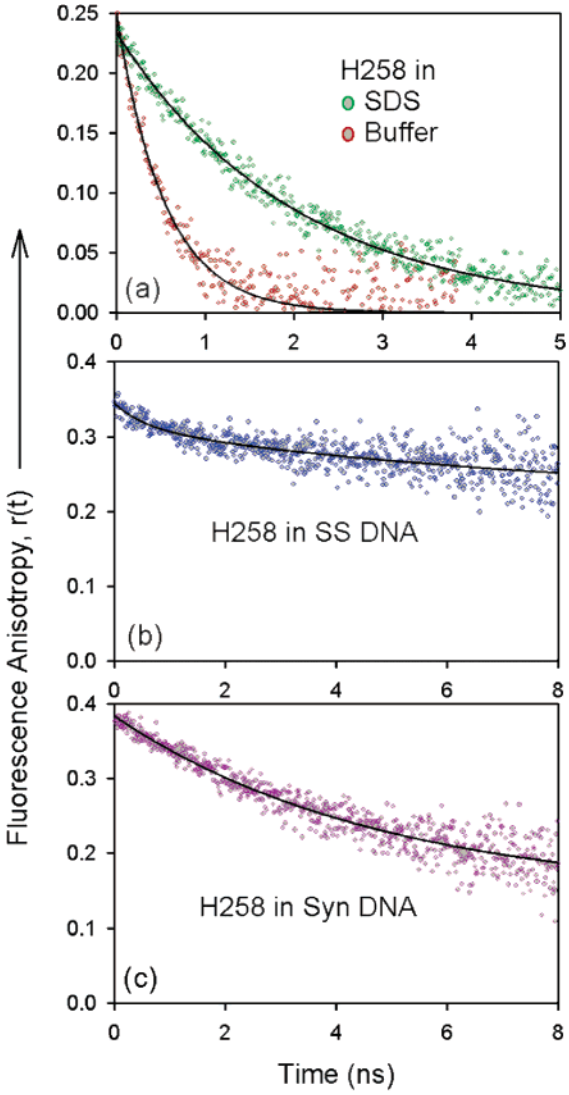

Figure 2. Fluorescence anisotropy decays of 1 μM H258 in 50 mM phosphate buffer, 50 mM SDS, 100 μM (base pair) SS DNA, and 70 μM (base pair) dodecamer.

effective technique to find out the distance between two ligands having overlap of their emission and absorption spectrum. We studied the resonance energy transfer between the ligands H258 and EtBr in SDS micelles. In the micellar system, the donor and acceptor molecules can be bound simultaneously without any restriction on the relative orientation of their transition dipole moments. Thus, the orientation parameter (κ^2) can be taken as 0.667.[31] Figure 3a shows that there is sufficient spectral overlap between the emission spectrum of the H258 (donor) and the absorption spectrum of the EtBr (acceptor) in SDS micelles. In order to prevent homomolecular energy transfer between donor molecules and to ensure efficient energy transfer between the donor and acceptor, the concentration of the donor molecules is kept low and that of the acceptor molecules is comparable to the micellar concentration. Both H258 and EtBr occupy the micellar interface,[28] and the relative orientation of the bound ligands is random in the SDS micelles. The energy transfer takes place from the donor to the acceptor, as indicated by the quenching of fluorescence intensity (Figure 3b) as well as the faster decay (Figure 3c) of the donor in the donor−acceptor complexes in micelles compared to that of only donor in the micelles. Analyses of the above-mentioned temporal fluorescence decays show that 12% of the donor molecules bound to the micelles are not involved in energy transfer. The population may be reflective of the donor molecules in the micelles without any acceptor. Our studies also reveal that 62% of the donor molecules undergo energy transfer with an energy transfer efficiency of 96.67% and the remaining 26% with an efficiency of 75.24%. The distances between the donor and the acceptor have been estimated to be 2.07 and 3.02 nm, by using an R_0 value of 3.67 nm. The observation is consistent with the binding of the donor and acceptor across the chords in the spherical SDS micelle (≈4 nm diameter[34]).

Figure 4a shows the spectral overlap between the absorption and emission spectra of the acceptor and the donor, respectively, in 100 μM salmon sperm DNA. The concentration of the EtBr (10 μM) has been chosen so as to ensure maximum intercalation of the dye (considering one ethidium molecule intercalates per 10 base pairs[21]). On the addition of acceptor (EtBr) molecules to H258−DNA solution, there is no shift in the emission maxima of the probe H258 compared to that of the H258−DNA complex without EtBr, indicating that the donor is still bound to the DNA. The binding of the ethidium molecules to DNA is confirmed by the 22 ns component in the temporal fluorescence decay characterizing the DNA environment[21] (inset of Figure 4c). Circular dichroism spectra (data not shown) show that the simultaneous binding of these two ligands does not alter the average secondary structure of the native DNA. The quenching of the fluorescence intensity (Figure 4b) coupled with the appearance of faster components in the decay (Figure 4c) of the H258 in the presence of EtBr in the DNA suggests considerable energy transfer from the donor to the acceptor molecule. It has been suggested in a previous study[12] that the donor and acceptor molecules in the genomic DNA cannot assume random orientations with respect to each other.[12] Thus,

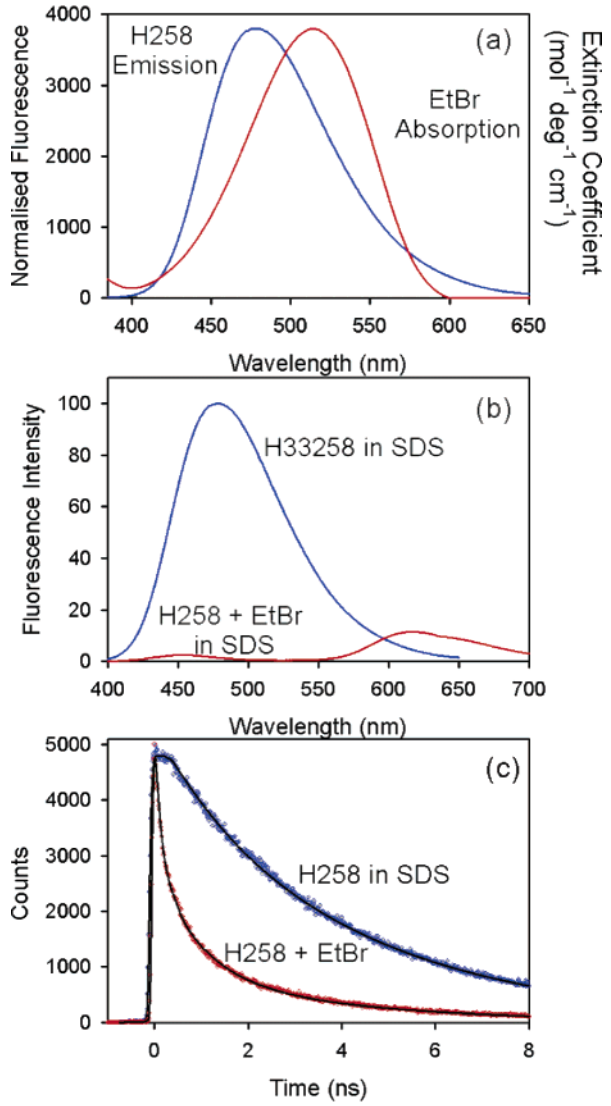

Figure 3. (a) Spectral overlap of H258 and EtBr in 50 mM SDS (~700 μM micellar concentration). The emission spectrum (b) and the temporal decay (c) of H258 (1 μM) and H258−EtBr ([EtBr] = 700 μM) in 50 mM SDS.

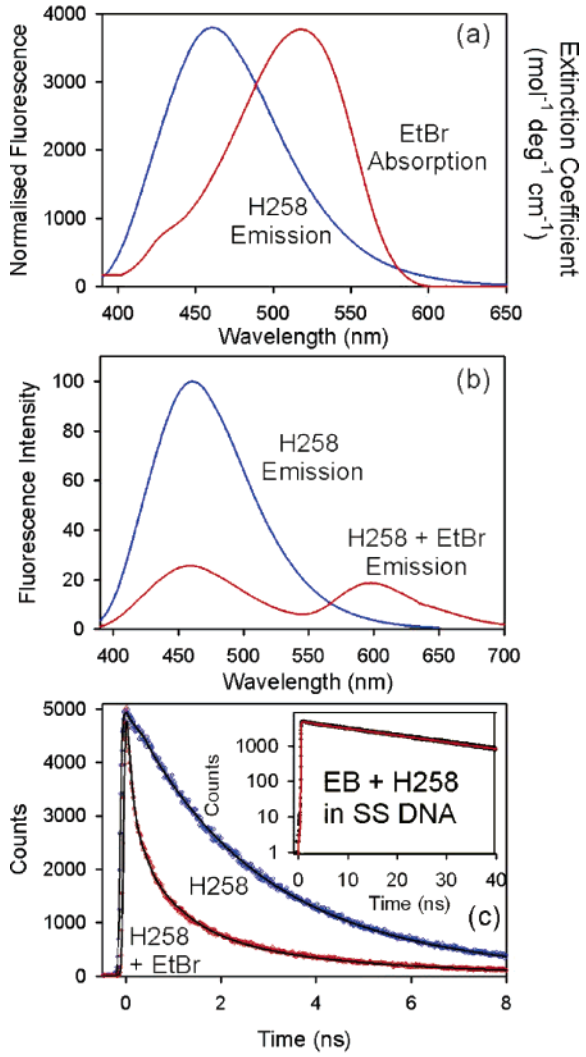

Figure 4. (a) Spectral overlap of H258 and EtBr in 100 μM (base pair) genomic DNA. The emission spectrum (b) and the temporal decay (c) of H258 (1 μM) and H258−EtBr ([EtBr] = 10 μM) in genomic DNA.

the value of κ^2, which takes into account the relative orientation of the donor and acceptor transition dipoles,[31] cannot be taken as 0.667, the value in the random orientation condition. In accordance with the above-mentioned study, the calculated value of R_0, using a κ^2 value of 1.2, is found to be 3.23 nm. Analyses of the temporal decays of the donor and the donor−acceptor complex in the genomic DNA show that 5% of the DNA-bound donor is not involved in energy transfer, 51% transfers energy to the acceptor with an efficiency of 97.75%, 25% transfers energy to the acceptor with an efficiency of 84.55%, and the remaining 17% undergoes energy transfer with an efficiency of 50.79%. The corresponding distances are estimated to be 1.77, 2.50, and 3.30 nm. It has been shown that the center of the H258 (donor) is situated at a distance of 0.4 nm from the helix axis.[35] The probability of energy transfer between donor and acceptor molecules bound to different DNA strands (inter-DNA energy transfer) has been checked by a control experiment. In the experiment, two separate solutions, one containing the donor (H258) bound to genomic DNA and another containing the acceptor (EtBr) bound to genomic DNA, are mixed. The temporal decay of the resultant solution shows no faster component associated with energy transfer. The result indicates that there is no inter-DNA energy transfer. Using this information along with the above-mentioned donor−acceptor distances, it is estimated that the centers of the H258 and EtBr molecules are separated by 5, 7, and 10 base pairs, respectively, within the persistence length of the genomic DNA. A donor−acceptor distance of 3.30 nm can also be assigned to donors and acceptors coming in close proximity due to folding and loop formation in genomic DNA.[36] Therefore, this study does not conclude that the donor and acceptor molecules are bound to the same region of the genomic DNA.

In order to verify whether the intercalator EtBr and groove binder H258 can bind to the same region of the DNA, the FRET studies are carried out in the dodecamer DNA. Each of the dyes individually binds to the dodecamer, as shown in separate studies.[7,21] The X-ray crystal structure of H258 bound to the minor groove of the dodecamer shows that the probe binds to the central A−T rich sequence involving five base pairs.[7] In a solution containing both the dyes H258 and EtBr in dodecamer DNA, the dye H258 shows emission maxima at 460 nm, characteristic of minor groove binding (Figure 5b), whereas the dye EtBr shows the 22 ns component at 620 nm, indicative of intercalation[21] (inset of Figure 5c). Figure 5a shows the spectral overlap between the emission spectra of the donor and the absorption spectra of the acceptor in the dodecamer. The quenching of fluorescence intensity (Figure 5b) along with the

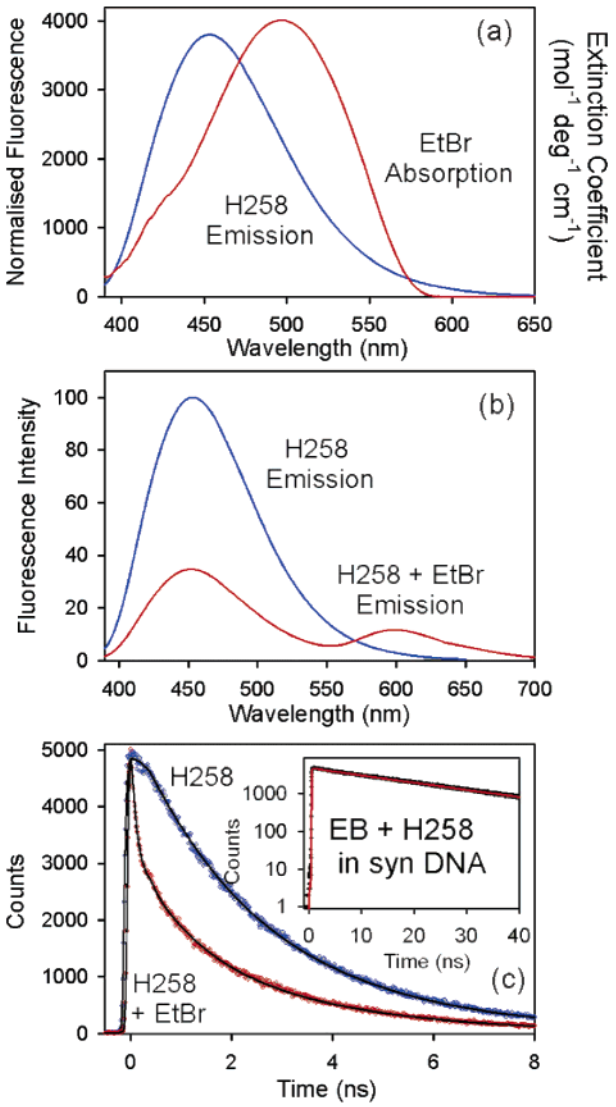

Figure 5. (a) Spectral overlap of H258 and EtBr in 70 μM (base pair) synthesized DNA ([DNA] = 5.8 μM). The emission spectrum (b) and the temporal decay (c) of H258 (1 μM) and H258−EtBr ([EtBr] = 6 μM) in synthesized DNA.

faster temporal decay (Figure 5c) in the H258−EtBr complex relative to that of the H258 in the dodecamer suggests energy transfer between the two molecules.

The binding possibility of the intercalator and the minor groove binder to different DNA molecules has been carefully avoided using an ethidium concentration equal to that of the DNA concentration, indicating that the energy transfer takes place within the same DNA. The X-ray crystallographic studies[7] (solid phase of DNA) along with NMR studies[25] (concentrated solution) on the dodecamer do not report any aggregation or hairpin structure formation. The structure of the dodecamer in the native state and in the presence of both of the dyes has been studied by CD spectroscopy. The results (data not shown) show that the simultaneous binding of the two dyes does not bring about a major perturbation to the structure of the dodecamer. The possibility of the energy transfer between the dye molecules bound to different dodecamer units can be ruled out considering that there is no homomolecular energy transfer[37] between the H258/EtBr molecules bound to the dodecamer. To further confirm that the energy transfer takes place between the dye molecules bound to a single dodecamer, a control experiment is performed. As a control, two separate solutions, one containing the donor (H258) bound to dodecamer DNA and another containing the acceptor (EtBr) bound to dodecamer DNA, are mixed. The temporal decay of the resultant solution shows no faster component associated with energy transfer. The result confirms that the energy transfer is indeed intra-DNA.

The difference between the temporal decays of the H258−EtBr complex in genomic (Figure 4c) and dodecamer (Figure 5c) DNA clearly points out the difference in binding of these two dyes to the different types of DNA. This difference could be due to the fact that the relative orientations of the transition dipoles of the donor and the acceptor are different in genomic and synthetic DNA. In the synthesized DNA, the molecules H258 and EtBr attain a definite geometry relative to each other. The transition dipole moment of EtBr is inclined 75° with respect to the helix axis.[10,18,38] The transition dipole of the minor groove binding drug, H258, is perpendicular to the long axis of the minor groove, which in turn makes an angle of 51° with the helix axis. The transition dipoles of the donor−acceptor pair thus make an angle of 66° with respect to each other. Using these results, the value of κ^2 was estimated to be 0.04 by using eq 2 and the R_0 value was calculated to be 1.91 nm. It is calculated that 21% of the donor molecules are not involved in energy transfer. The loss of efficiency of FRET of the donor in the close proximity of the acceptor in the dodecamer is a clear indication of a smaller value of κ^2 compared to those in the genomic DNA and SDS micelles. From our studies, it is also clear that 60% of the donor molecules show an energy transfer efficiency of 96.47% to the acceptor situated at 0.92 nm (two base pairs away from the donor), whereas the remaining 17% transfer energy to an acceptor located at 1.95 nm with an efficiency of 50.12%. The distance of 1.95 nm, which is five base pairs away from the acceptor, reveals that the acceptor molecules are intercalated at the ends of the dodecamer. The small percentage of intercalator binding to the ends (five base pairs from the donor) is consistent with other studies.[39] It is worthwhile to mention that if the value of κ^2 is taken as 1.2, the calculated donor−acceptor distances are 1.92 and 3.37 nm. The distance of 3.37 nm indicates that the H258 and EtBr are separated by a distance of 10 base pairs. The result is unphysical because in the dodecamer DNA the maximum distance from the center of the helix is 2.07 nm (six base pairs). Considering the random orientation of the transition dipoles of the donor−acceptor ($\kappa^2 = 0.667$), the calculated donor−acceptor distance of 3.06 nm also has no physical significance.

Conclusion

Our studies on the dodecamer DNA show that the minor groove binding by H258 and intercalation by EtBr can independently take place involving a particular site of the dodecamer. The intercalator and the minor groove binder on simultaneous binding to dodecamer DNA have their transition dipoles oriented at 66° with respect to each other. In the dodecamer, the majority of the acceptor molecules are located at a distance of 0.92 nm from the donor (H258) in the center of the DNA, and a small number of acceptors are intercalated in the ends of the dodecamer at a distance of 1.92 nm. The binding nature is essentially different from that of the two dyes bound to genomic DNA, where the two dyes are separated by five and seven base pairs along the persistence length or by loop formation by a distance of 3.3 nm. It is also revealed that the use of the calculated value of the orientation parameter ($\kappa^2 = 0.04$) is crucial for the estimation of the distance between the donor and acceptor bound to the dodecamer. The use of the value of the orientation parameter for the random distribution of acceptor

($\kappa^2 = 0.667$) as well as that of partially restricted distribution of acceptor ($\kappa^2 = 1.2$) lead to erroneous results.

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