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Simultaneous Binding of a Cyclophane and Classical Intercalators to DNA: Observation of FRET-mediated White Light Emission

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DNA-assisted Förster Resonance Energy Transfer (FRET) between an anthracene-based cyclophane (CP) and mono- and bis-intercalators such as propidium iodide (PI) and ethidium homodimer-1 (EHD), respectively, have been studied using various photophysical and biophysical techniques. The cyclophane and PI exhibited simultaneous binding to DNA at all concentrations studied and showed DNA-assisted FRET from the excimer of cyclophane with a FRET efficiency of ca. 71%. On the other hand, the bis-intercalator EHD, only at lower concentrations (< 3 µM) can act as an acceptor for the energy transfer process with a lower efficiency of ca. 44%. At higher concentrations (> 15 µM), EHD on account of its higher binding affinity, displaces cyclophane from the DNA scaffold. Employing the ternary system comprising of the cyclophane, DNA and PI and fine-tuning the concentrations of the components in the molar ratio of 1:0.75:0.05 (CP:DNA:PI) we have demonstrated white light emission with CIE coordinates (0.35, 0.37).

INTRODUCTION

The development of organic white light emitting materials hold great promise for the production of highly efficient, large area light sources.1, 2 FRET based fluorescence colour tuning, with fluorophores incorporated in various scaffolds including polymers, biomolecules, micelles, supramolecular assemblies and nanoparticles, is a widely investigated approach for white light generation.3-18 Among these scaffolds, DNA-cationic surfactant complexes have emerged as intriguing candidates for optoelectronic applications in recent years because of their unique material properties, including solubility in organic solvents and ability to form thermally stable, transparent films.18-24 Moreover, the predictable secondary structure, unique binding sites for small molecules and the ability to control the spatial arrangement of chromophores with nanoscale precision, makes DNA an excellent scaffold for related applications in optoelectronic devices and sensors.5, 25-28

A key component of developing DNA based energy transfer systems is the control of distances between the bound molecules, which has important role in the DNA mediated energy transfer process.27, 29-33 This in turn is regulated by apparent binding constants and allosteric factors when multiple chromophores are involved.33,44 While many groups have used the interaction of chromophores with DNA in demonstrating DNA mediated white light emission,18-23, 45 there exist need of fundamental understanding on the structure-property correlation controlling the output of the light emission.

In this context, we have started investigating the effect of simultaneous binding of ligands, which occupy orthogonal binding sites on the DNA helix, on FRET related optical properties. For example, We have recently demonstrated the simultaneous binding of an anthracene-based cyclophane (CP), a non-classical intercalator and a DNA intercalator, ethidium bromide (EB) with potential for emission colour tuning (Fig. 1).46 The symmetric cyclophane exhibited dual emission in aqueous medium having monomer emission maximum at 430 nm and an intramolecular excimer at 550 nm.47, 48 In presence of DNA, the excimer emission got enhanced with concomitant quenching of monomer emission. Interestingly, in presence of ethidium bromide, we could observe an efficient FRET from the cyclophane to the intercalated ethidium bromide. Finally, we used this ternary system for the demonstration of white light emission by appropriately changing the concentrations of the individual constituents.46

Herein, we have investigated the role of two different intercalator energy acceptors, namely ethidium homodimer-1, a
known bis-intercalator and propidium iodide, a mono-intercalator with better binding affinity than the earlier reported ethidium bromide and compared their optical properties. Our results, demonstrated that the bis-intercalator, with three order higher DNA binding affinity tend to displace the cyclophane from the DNA scaffold at comparable concentrations while propidium iodide showed a better FRET efficiency of ca. 71%, compared to that of ethidium bromide.

**GENERAL EXPERIMENTAL TECHNIQUES**

Fluorescence spectra were recorded, in 1×1 cm quartz cuvettes, on a SPEX–Fluorolog F112X spectrofluorimeter. CIE, Commission Internationale de l’Eclairage (International Commission on Illumination), chromaticity coordinates (1931) (x, y) in thin films were calculated using HORIBA Jobin Yvon Color Calculator provided with integrating sphere. Fluorescence lifetimes were measured using IBH (FluoroCube) time-correlated picoseconds single photon counting (TCSPC) system. The thin films were excited with a pulsed diode laser (<100 ps pulse duration) at a wavelength of 375 nm (NanoLED-11) with a repetition rate of 1 MHz. The detection system consisted of a microchannel plate photomultiplier (5000U-09B, Hamamatsu) with a 38.6 ps response time coupled to a monochromator (5000M) and TCSPC electronics [data station Hub including Hub-NL, NanoLED controller, and preinstalled fluorescence measurement and analysis studio (FMAS) software]. The fluorescence decay profiles were deconvoluted using IBH data station software V2.1 and minimizing the $\chi^2$ values of the fit to 1 ± 0.1. The quantum yield of fluorescence of the cyclophane CP in presence of DNA was calculated using equation (1).49

$$\Phi_u = \frac{A_F \Phi_u}{A_u \Phi_s} \Phi_s$$

where, $A_u$ and $A_o$ are the absorbance of standard and unknown, respectively. $F_s$ and $F_u$ are the areas of fluorescence peaks of the standard and unknown and $n_i$ and $n_o$ are the refractive indices of the solvents used for the standard and unknown, respectively. Quinine sulfate ($\Phi_s = 0.546$) was used as the standard for determination of fluorescence quantum yields.50, 51

Solvents and reagents were purified and dried by usual methods prior to use. Dried methanol was used for all the studies. All experiments were carried out at room temperature (25 ± 1 °C), unless otherwise mentioned.

**Materials**

Calf thymus (CT) DNA, Propidium iodide and ethidium homodimer-1 were purchased from Sigma–Aldrich and used as received. A solution of CT DNA was sonicated for 1 h to minimize the complexities arising from DNA flexibility and filtered through a 0.45 μm Millipore filter ($M_o = 3 \times 10^6$ gmol$^{-1}$). The concentrations of DNA solutions were determined by using the average extinction coefficient value of 6600 M$^{-1}$cm$^{-1}$ of a single nucleotide at 260 nm. CP was prepared according to the literature procedure.47, 48

Calculation of FRET efficiency and donor-acceptor distance

To calculate the efficiency of FRET and the distance between the donor and acceptor moieties equation (2) was used.

$$R_0 = 0.211[\kappa^2 n_2 Q_D J(\lambda)]^{1/6}$$

where $R_0$ is the Förster distance, $\kappa^2$ is a factor describing the relative orientation in space of the transition dipoles of the donor and acceptor and assumed to be equal to 1.25.53-55 $n$ is the refractive index of the medium and assumed to be 1.3, $Q_D$ is the quantum yield of the donor in the absence of acceptor and $J(\lambda)$ is the overlap integral, which expresses the degree of spectral overlap between the donor emission and the acceptor absorption given by equation (3).

$$J(\lambda) = \int_0^\alpha F_D(\lambda) d\lambda$$

where, $F_D(\lambda)$ is the fluorescence intensity of the donor in the wavelength range of $\lambda$ to $\lambda + d\lambda$ and $\alpha(\lambda)$ is the extinction coefficient (in M$^{-1}$cm$^{-1}$) of the acceptor at $\lambda$. The orientation factor ($\kappa^2$), we assumed a value of 1.25 instead of 0.67,53-55 since the DNA-bound molecules are not in random orientation. From the value of $R_0$ obtained from equation 2, the donor–acceptor distance ($r$) can be calculated using equation (4).

$$r^6 = \frac{R_0^6 (1 – E)}{E}$$

where, E is the efficiency of energy transfer, which is calculated from the lifetimes of the donor in the absence and presence of acceptor ($\tau_D$ and $\tau_{DA}$) as per the equation (5).

$$E = 1 – (\tau_{DA}/\tau_D)$$

**RESULTS AND DISCUSSION**

**FRET between CP and DNA intercalators**

Ethidium bromide (EB), propidium iodide (PI) and ethidium homodimer-1 (EHD) (Fig. 1) are phenanthridinium derivatives, which exhibit weak fluorescence in aqueous medium and their fluorescence intensities were known to be enhanced in the presence of DNA.56 The DNA mono-intercalators EB and PI bind to DNA with association constants of 1.4 x 10$^5$ M$^{-1}$ and 8.6 x 10$^5$ M$^{-1}$, respectively.44 On the other hand, EHD, which contains two phenanthridinium moieties was found to undergo bis-intercalation with an association constant of 2 x 10$^6$ M$^{-1}$.40, 41, 43 The anthracene-based cyclophane (CP) interacts with DNA through non-classical partial intercalation and exhibit a bathochromically shifted excimer emission, which can be utilized for energy transfer to suitable acceptor molecules.44, 48 The excimer emission at 570
nm has been confirmed by recording the excitation spectra of the cyclophane with and without DNA and they are found to be similar to the corresponding absorption spectra (Fig. S1†). First we investigated the utility of the dicationic molecule PI as the excited state energy acceptor in the FRET process. The absorption and emission changes observed with PI are similar to that of EB (Fig. S2-S3†), though the binding affinity of PI towards DNA is slightly higher. The calculated spectral overlap between the cyclophane excimer emission and PI absorption are found to be higher than that with EB. The energy transfer efficiency between CP and PI was calculated to be ca. 71% as against 62% with EB. Hence the FRET between CP and PI was found to be higher and the calculated donor-acceptor distance is 1.7 nm.

![Fig. 1 Structures of molecules used for the study.](image)

In order to understand how the bis-intercalator behaves in the energy transfer process, we monitored the absorption and emission parameters of the cyclophane in presence of EHD. At lower concentrations of the intercalator, we observed the excitation energy transfer to the acceptor molecule (Fig. 2 & Fig. S4†). Interestingly, when we further increase the concentration to 15 µM it was observed that there is a gradual quenching of the acceptor fluorescence. The absorption changes also complement this observation. At 15 µM concentration of EHD, we observed ca. 38% hyperchromicity in the anthracene absorption region, indicating thereby the less significant interactions between CP and DNA. Moreover, at 15 µM concentrations of EHD, the system exhibited an emission spectrum, which is similar to that of the free cyclophane in buffer medium. An explanation for these observations is based on the fact that at these higher concentrations, the bis-intercalator, EHD found to displace CP from the DNA template on account of its higher binding affinity.

**Fluorescence lifetime studies and time resolved spectroscopy**

To further understand the FRET between CP and phenanthridinium derivatives in the presence of DNA, we investigated the fluorescence lifetimes of CP under different conditions. CP in the aqueous medium exhibited a bi-exponential decay with lifetimes of 13.4 and 52.6 ns, which can be assigned to monomer and excimer, respectively. Interestingly, in the presence of DNA we observed significant enhancement in the excimer lifetime with a concomitant decrease in the monomer lifetime. For example, the excimer lifetime of CP in the buffer medium (52.6 ns) has been enhanced to 143.1 ns, in the presence of 38 µM DNA. Then we investigated the role of FRET acceptors such as PI and EHD in the fluorescence lifetime of cyclophane excimer. For example, at 8 µM concentration of PI, a bi-exponential decay with lifetimes of 55.7 and 20.2 ns was observed, wherein the long-lived species correspond to the cyclophane excimer. This reduction in excimer lifetime from 143.1 to 55.7 ns can be attributed to the effective excitation energy transfer from CP to PI (Fig. 4A).

The time-resolved emission spectroscopic (TRES) technique was utilized further to understand FRET process. When a system containing CP, DNA and PI was excited at 380 nm and analyzed immediately after excitation (2 ns), we observed an emission spectrum with maximum at 430 nm and a small shoulder at 625 nm (Fig. S5†). However, gradually with time, the intensity of the peak at 625 nm was found to increase with the complete disappearance of monomer peak at 430 nm.

![Fig. 2 Changes in the emission spectra of DNA-bound CP by the addition of EHD in phosphate buffer. (CP), 16 µM; (DNA), 27 µM; (EHD), (a) 0 and (g) 15 µM. Excitation wavelength, 380 nm.](image)
Importantly, the absence of the emission band at 570 nm corresponding to the excimer of CP in the presence of PI at various time scales points to the fact that its formation is quenched due to the existence of an efficient FRET from CP excimer to PI. After 60 ns, the system exhibited a broad peak at 625 nm, corresponding to PI through FRET from CP. Thus, at longer time scale, we could observe emission from the acceptor only, which is a clear confirmation for the existence of excited-state energy transfer from the excimer of CP to PI (Fig. S5†).

With the bis-intercalator EHD at 3 µM concentrations, the system showed a bi-exponential decay with lifetimes of 93.2 and 22.2 ns. The reduction of excimer lifetime from 143.1 ns to 93.2 ns in presence of EHD is an indication of FRET from CP to the intercalated EHD (Fig. 4B). Further evidence was provided through the TRES studies with 3 µM concentrations of the bis-intercalator EHD, wherein we observed the cyclophane monomer emission at 430 nm immediately after excitation (Fig. S6†). But at longer time scale (60 ns), we observed an emission band around 620 nm, which corresponds to EHD. However, at 15 µM concentrations of EHD, the system exhibited bi-exponential decay with lifetimes of 16 ns and 52.8 ns, when monitored at 550 nm. These lifetime values correspond to the monomer and excimer emissions of free CP in the aqueous medium (Fig. S7A†). These experiments once again confirmed that at higher concentrations (ca. 15 µM), EHD displaces CP from the DNA scaffold thereby ruling out the possibility of FRET under these conditions. In the TRES analysis, at higher concentrations of EHD, the spectrum immediately after excitation (2 ns) exhibited two peaks at 430 and 625 nm, corresponding to cyclophane monomer emission and energy transferred emission from EHD. However, with time, we could observe reduction in the intensity of EHD emission and the observation of the formation of a new peak around 540 nm, which corresponds to the excimer emission of the cyclophane in the buffer medium (Fig. S7B†).

Based on the fluorescence and lifetime data, we calculated the efficiency of energy transfer between various donor-acceptor pairs and the corresponding donor-acceptor distances. The energy transfer efficiency between CP and PI was found to be ca. 71% whereas, the FRET efficiency between CP and 3 µM EHD was found to be ca. 44%. The donor-acceptor distances between CP-PI and CP-EHD systems were found to be ca. 17 and 24 Å, respectively (Table 1). The rate of FRET in various donor-acceptor systems were also calculated and tabulated (Table 1).

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Rate of energy transfer ((10^{-12} \text{ s}^{-1}))</th>
<th>Forster distance (Å)</th>
<th>D-A distance (Å)</th>
<th>FRET efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHD†</td>
<td>0.15</td>
<td>18.7 ± 0.5</td>
<td>24 ± 0.5</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>PP†</td>
<td>1.67</td>
<td>19.7 ± 0.2</td>
<td>17 ± 0.2</td>
<td>71 ± 1</td>
</tr>
<tr>
<td>EB†</td>
<td>1.14</td>
<td>34.2 ± 0.1</td>
<td>31.5 ± 0.1</td>
<td>62 ± 1</td>
</tr>
</tbody>
</table>

*Average of three independent measurements. †Data taken from ref. 46

**Demonstration of white light emission**

To demonstrate white light emission in aqueous medium, we utilized the FRET between CP and PI in presence of DNA since the energy transfer efficiency between CP and PI was found to ca. 71%. This can be achieved by taking the individual components in a proper ratio so that the emission spectrum can cover the entire visible region (400-700 nm). In this context, we have taken CP, DNA and PI in the molar ratio of 1:0.75:0.05 in 3 mL of the aqueous medium, and recorded the emission spectrum upon 380 nm excitation. The observed emission spectrum covered the entire visible region, wherein, the blue region was contributed by the emission from the cyclophane monomer (at 430 nm), green region by the cyclophane excimer (at 560 nm) and the red region by the DNA intercalated PI molecule (at 620 nm) (Fig. 5). We have calculated the CIE coordinates of the emission spectrum which are found to be (0.35, 0.37). These values are very close to the CIE coordinates of pure white light emission (0.33, 0.33). It is interesting to note that the amount of the acceptor molecule required in this case was quite low (ca. 2 mol%) because of the existence of an efficient FRET process.
CONCLUSIONS

In conclusion, we have investigated the interactions between the cyclophane CP and DNA intercalators such as PI and EHD using various photophysical techniques. In presence of DNA, we observed that these energy acceptors significantly quenched the excimer emission intensity of the cyclophane with the concomitant formation of new peak in the red region through FRET mechanism. The steady-state and time-resolved fluorescence emission studies have revealed that, these phenantroindium derivatives were efficient excited-state energy acceptors. Based on the energy transfer efficiency studies, the system containing CP and PI was found to be the best donor–acceptor pair, in comparison with the bis-intercalator EHD and the mono-intercalator EB and the system exhibited an energy transfer efficiency of ca. 71%. Using the CP–DNA–PI system in the molar ratio of 1:0.75:0.05 in 3 mL of aqueous medium, we obtained an emission spectrum with the CIE coordinates (0.35, 0.37), which correspond to pure white light emission. These studies further confirmed that both efficiency and nature of DNA binding, and the spectral overlap between donor and acceptor systems play significant role in the generation of DNA–assisted white light emission.

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Notes and references