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Sensing of DNA conformation based on change in FRET efficiency between laser dyes

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ABSTRACT

This communication reports the effect of DNA conformation on fluorescence resonance energy transfer (FRET) efficiency between two laser dyes in layer by layer (LbL) self assembled film. The dyes Acraflavine and Rhodamine B were attached onto the negative phosphate backbones of DNA in LbL film through electrostatic attraction. Then FRET between these dyes was investigated. Increase in pH or temperature causes the denaturation of DNA followed by coil formation of single stranded DNA. As a result the FRET efficiency also changed along with it. These observations demonstrated that by observing the change in FRET efficiency between two laser dyes in presence of DNA it is possible to detect the altered DNA conformation in the changed environment.

1. Introduction

By measuring the fluorescence signals it is possible to detect DNA hybridization where the dye molecules are intercalated into a DNA double helix [1,2]. But the inherent limitation of this method is the lack of specificity for many particular duplex [3,4]. Another most important strategy for the detection of DNA hybridization involves fluorescence resonance energy transfer (FRET). FRET between two molecules is an important physical phenomenon, where transfer of energy occurs from an excited donor fluorophore to a suitable acceptor fluorophore [5,6]. Combining FRET with optical microscopy, it is now possible to determine the distance between two molecules in nanometers. The basic requirements for the FRET to occur are (i) sufficient overlap between the absorption band of acceptor fluorophore and the fluorescence band of donor fluorophore and (ii) both the donor and the acceptor molecule must be in close proximity of the order of 1–10 nm [5,6]. The interference of solvent or other macromolecules has little effect on the FRET efficiency [7–9]. Literature survey suggests that FRET process can be used to investigate molecular mechanisms [10,11] as changes in the distance between the donor and the acceptor molecules effect the FRET efficiency.

Double stranded DNA is an interesting anionic polyelectrolyte with unique double helix structure whose base sequence controls the heredity of life [12]. DNA, the eternal molecule shows autocatalytic property i.e., self replication. The process involves the partial separation of two individual strands known as denaturation of DNA.

During annealing and thermal denaturation of duplex DNA the donor–acceptor moieties are brought closer together or moved further apart and as a result changes occur in the fluorescence intensity of the FRET pair. Using this principle various researchers reported the detection of target DNAs by excimer–monomer switching of Pyrene and by DNA based nano-device [7–9].

The denaturation of DNA can also be done by changing the hydrogen ion concentration of the medium. Though extensive researches were carried out in the field of thermal denaturation of DNA [12–14] comparatively less attention has been paid in alkaline denaturation of DNA [15–17]. The stability of a duplex DNA has a relation with environmental pH [17]. Increase in alkaline pH level of the cells may cause denaturation of DNA which eventually affects gene expression.

The present communication focuses on the changes in FRET efficiencies between two dyes Acriflavine (Acf) and Rhodamine B (RhB) associated in LbL films in presence of double stranded DNA as well

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as denatured DNA. In the present study, the denaturation of the DNA has been studied in a wide range of changes in pH and temperature. This technique has potential applications in optoelectronic and thin film devices [18,19] and is also important for better understanding of some biological systems. The present investigation is, therefore, aimed at detection of altered DNA conformation in the changed environment which could be used in logic gates operations, sensing purpose, nano transporting and above all in nano medicine [20–23].

2. Materials and methods

2.1. Materials

The purity of Salmon sperm DNA (SRL India) was checked by UV–vis absorption and fluorescence spectroscopy before use. All other reagents viz., Acf, RhB, poly acrylic acid (PAA) and poly allylamine hydrochloride (PAH) were purchased from Sigma Chemical Co., USA for experimental purposes. Ultrapure Milli-Q water (resistivity 18.2 MΩ cm) was used as solvent.

2.2. Film preparation

Electrolytic deposition bath of cationic dye RhB and Acf were prepared with 10^{-4} M aqueous solution using triple distilled deionized Millipore water. The anionic electrolytic bath of PAA was also prepared with triple distilled deionized Millipore water (0.25 mg/mL). LbL self assembled films were obtained by dipping clean fluorescence grade quartz substrate first in the solutions of anionic PAA for 30 min. Then it was taken out and sufficient time was allowed for drying. It was followed by thorough rinsing in water bath for 2 min so that the surplus anions attached to the surface were washed off. The dried substrate was then immersed in to the cationic dye mixture of RhB and Acf (1:1) followed by same rinsing procedure. Deposition of PAA and RhB – Acf layers resulted in one bi-layer of self assembled film. The incorporation of DNA into the LbL film was done with the help of aqueous PAH solution (0.25 mg/mL). For each experimental set, the quartz slide was first dipped in to the electrolytic aqueous solution of polycation (PAH) for 30 min followed by same rinsing in water bath and drying procedure and then dipped into the anionic DNA (con. = 0.25 mg/mL) solutions of different pH (5–13.5) which was again followed by rinsing action in water bath. NaOH and HCl were used to increase and decrease the pH of DNA solution. The slide thus prepared was dipped into the cationic electrolytic solution of RhB and Acf (1:1). Due to electrostatic interaction, cationic Acf and RhB were adsorbed onto the negatively charged surface of the DNA in LbL films. LbL method utilizes the Van der Waals interactions between the quartz slide and PAA as well as electrostatic interaction between PAA and cationic dyes [24].

2.3. UV–vis absorption and fluorescence spectra measurement

UV–vis absorption and fluorescence spectra were recorded by a Perkin Elmer Lambda-25 Spectrophotometer and Perkin Elmer LS-55 Fluorescence Spectrophotometer respectively. For absorption measurement the LbL films were kept perpendicular to the incident light and fluorescence from the sample surface at an angle of 45° (front face geometry) was recorded. All the spectra were recorded with excitation wavelength at 420 nm.

2.4. AFM measurement

Atomic force microscopy (AFM) image, in intermittent contact (tapping) mode, of one bi-layer LbL film was taken in air with commercial AFM system (Bruker Innova). Typical scan area was 1 μm × 1 μm. The Si-wafer substrate was used for the AFM measurement.

3. Results and discussions

3.1. FRET between Acf and RhB in presence and absence of DNA

Acf and RhB dyes are highly fluorescent and, in principle suitable for FRET [25]. The fluorescence spectrum of Acf sufficiently overlaps with the absorption spectrum of RhB. There are few reports on the investigation of FRET between these two dyes [25]. In our laboratory we have also studied FRET phenomenon using these two dyes [26]. The absorption and fluorescence spectra of Acf and RhB in LbL films (Fig. S1) suggest that both the dyes remain as monomers in the LbL films [25,27]. The corresponding absorption and fluorescence maxima are shown in Table 1. Fig. 1 shows the fluorescence spectra of (i) pure Acf and RhB and, (ii) Acf–RhB mixed LbL films in presence and absence of DNA. The excitation wavelength was selected in order to excite the Acf molecules directly and to avoid any direct excitation of the RhB molecules. Acf shows strong fluorescence (curve 1 of Fig. 1) since it absorbs light in this excitation range. On the other hand, the RhB fluorescence (curve 2 of Fig. 1) is almost negligible. It is interesting to note that for Acf–RhB mixed LbL films (curve 3 of Fig. 1) the RhB fluorescence intensity increases and the Acf fluorescence intensity decreases with respect to their individual excitation behavior. It is likely that some energy is transferred from Acf to RhB which causes excitation of more RhB molecules followed by emission of light. Thus an increase in RhB fluorescence intensity and decrease in Acf fluorescence intensity was found. This has been confirmed by measuring the excitation spectra, where the monitoring emission maxima are 525 nm (Acf) and 580 nm (RhB) in case of Acf-RhB mixed LbL films. It has been observed that both the excitation spectra are almost similar (Fig. S2) and possess characteristic absorption bands of Acf monomers. This validates FRET between Acf and RhB. Further increase in RhB fluorescence and decrease in Acf fluorescence in Acf–RhB fluorescence spectra (curve 4 of Fig. 1) have been observed for the Acf–RhB mixed LbL films prepared in presence of DNA. This is an indication that the presence of DNA enhances the FRET. In one of our previous work we reported the influence of DNA in the FRET between Acf and RhB in solution phase and based on that we also developed a DNA sensor [28].

The FRET efficiencies were calculated from Fig. 1 using the equation given below [29]

$$E = 1 - \frac{F_{DA}}{F_D};$$

where F_{DA} is the relative fluorescence intensity of the donor in the presence of acceptor and F_D is the fluorescence intensity of the donor in the absence of the acceptor.

Our findings indicate that in presence of DNA, FRET efficiency of the dye pair increases from 28.42% to 44.62%. FRET is a distance dependent process and so, when inter molecular distance between donor and acceptor pair decreases the transfer of energy from donor to acceptor becomes more efficient [5,6]. Thus proximity of cationic Acf and RhB molecules in presence of DNA, by their electrostatic attachment to the phosphate moiety of negatively charged DNA, creates favorable condition for efficient energy transfer. This has been shown schematically in the later part of the manuscript.

3.2. Effect of pH on DNA and FRET

In order to investigate the effect of DNA denaturation on the FRET between Acf and RhB, we have prepared DNA LbL films at different pH and Acf–RhB mixed LbL films in presence of DNA at different pH. Fig. 2(a) shows the variation of absorbance intensity of the 260 nm band of DNA-PAH LbL films with increasing pH range

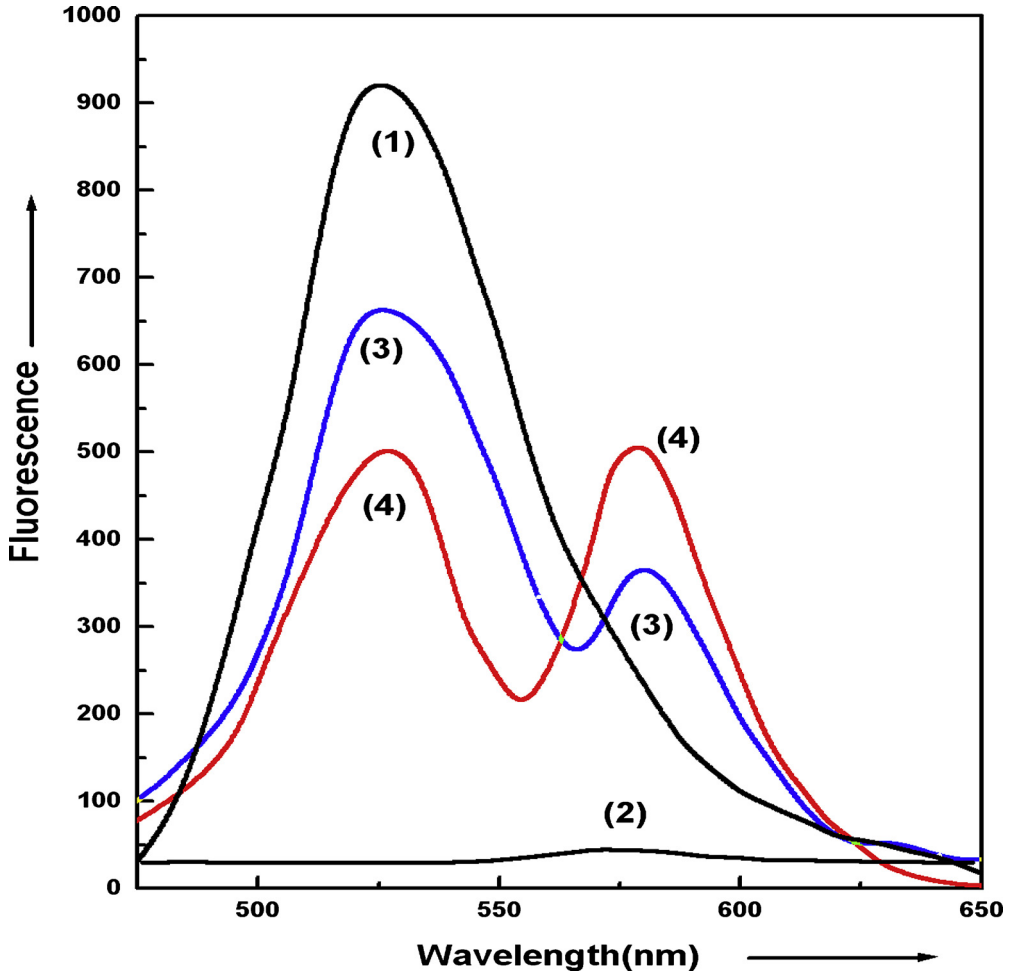

Fig. 1. Fluorescence spectra of pure Acf (1), pure RhB (2), Acf + RhB (3) and Acf + RhB with DNA (4) in LbL film.

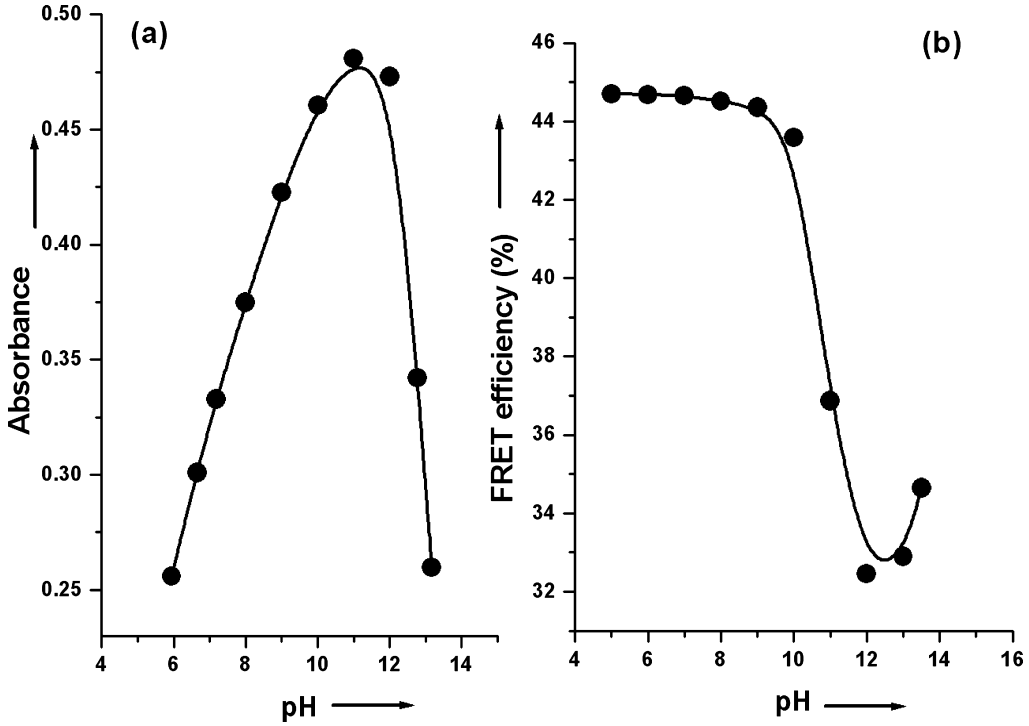

Fig. 2. (a) Variation of absorbance intensity of the 260 nm peak of the DNA–PAH LbL film prepared at different pH. (b) Variation of FRET efficiency of Acf + RhB LbL film in presence of DNA prepared at different pH.

Table 1
Absorption and fluorescence maxima of Acf and RhB in LbL films.

Absorbance		Fluorescence	
Acf	RhB	Acf	RhB
450 nm (monomer)	568 nm (monomer) 530 nm (0–1 vibronic)	525 nm (monomer)	580 nm (monomer)

Table 2
Physical parameters of Salmon sperm DNA.

DNA	Density (gm/cc)	G–C content (mol%)	Denaturation	Coil formation	Hypochrometic shift
Salmon sperm	1.703	42	pH = 11 Temp = 80 °C	pH = 13 Temp = 95 °C	43% (pH) 47% (Temp.)

from 5 to 13.5. From Fig. 2(a) it is observed that the absorbance intensity of 260 nm band of DNA-PAH LbL films increases gradually with increase in pH and at pH strength of near about 11 it becomes maximum. At higher pH the denaturation of DNA occurs that results in increase in absorbance intensity [12,30]. Based on the hypochrometic shift of DNA absorbance, denaturation and renaturation of DNA with pH variation have been reported earlier [12,30]. It is obvious that due to the increase in pH or temperature the H-bond in the double stranded DNA gets disrupted, the strands separate from each other and finally form individual random coil. The course of this dissociation can be followed spectrophotometrically, as the relative absorbance of the DNA–PAH–LbL films at 260 nm increases with DNA denaturation [12]. UV absorption of the bases in the DNA is due to the π-electron transition. In double stranded DNA the aromatic bases are stacked together and they interact via their p-electron clouds. This causes a decrease in π-electron transition probability. As a result the absorption intensity also decreases. On the contrary, due to unstacking of aromatic bases in single stranded DNA, the interaction between the p-electrons is less and this causes an increase in π-electron transition probability. Consequently, UV absorption intensity increases. At pH values greater than 10, extensive deprotonation of the bases occurs destroying their hydrogen bonding potential and denaturing the DNA duplex [31]. Above pH 12, a sharp decrease in the absorbance intensity suggests the coiling of single stranded DNA probably due to the ionization of the guanine moieties of the DNA [31]. The formation of water by the introduction of hydroxyl ions present in the solution with a proton of the H-bridges between the base pairs eventually releases some amount of energy during denaturation. This energy is mainly responsible for the coiling of single stranded DNA [32]. Concurrently, the length of transition dipole moment vector increases due to the breaking of H-bridges with increasing pH, which results in an increase in absorption intensity. The remarkable decrease in the length of the transition dipole moment vector is also an indication of formation of coiled single stranded DNA which reduces absorption intensity to a considerable extent.

Fig. 2(b) shows the variation of FRET efficiencies between Acf and RhB in the Acf–RhB LbL films in presence of DNA at different pH range from 5 to 13.5. It is observed that the transfer of energy from Acf to RhB in presence of DNA almost remains constant up to pH 9. This implies that the strands are not separated from each other i.e., the distance between Acf and RhB remains unchanged up to this pH. But from pH 10 to 12, the FRET efficiencies decrease remarkably indicating the separation between the strands of the DNA. Subsequent increase in pH after 12 results in some amount of increase in FRET efficiency between the Acf–RhB pair. This is

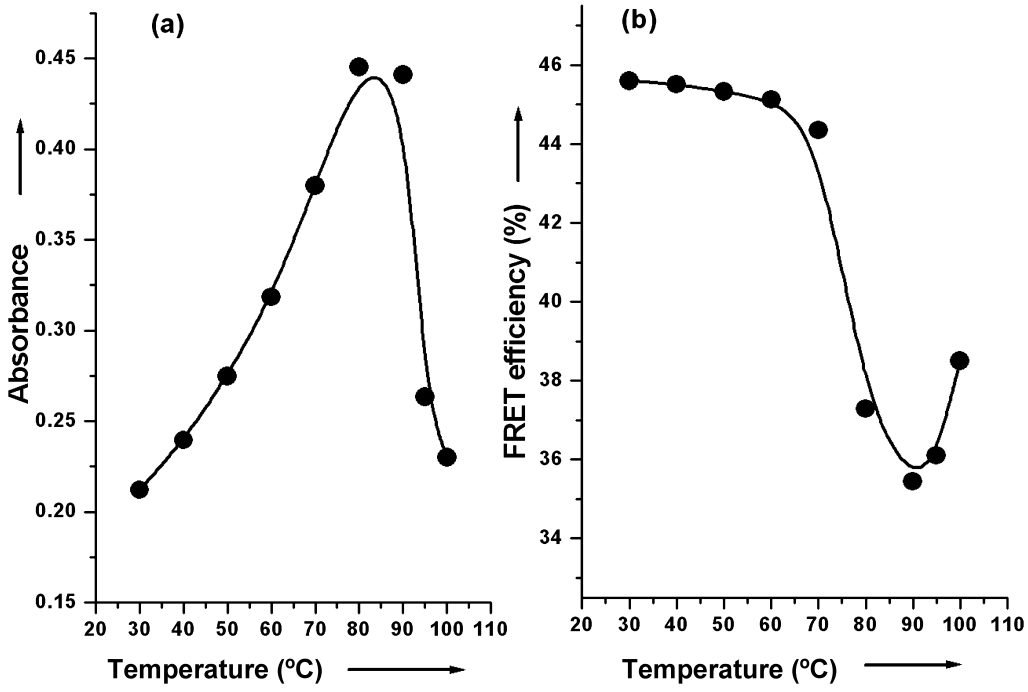

Fig. 3. (a) Variation of absorbance intensity of the 260 nm peak of the DNA–PAH LbL film prepared at different temperature. (b) Variation of FRET efficiency of Acf + RhB LbL film in presence of DNA prepared at different temperature.

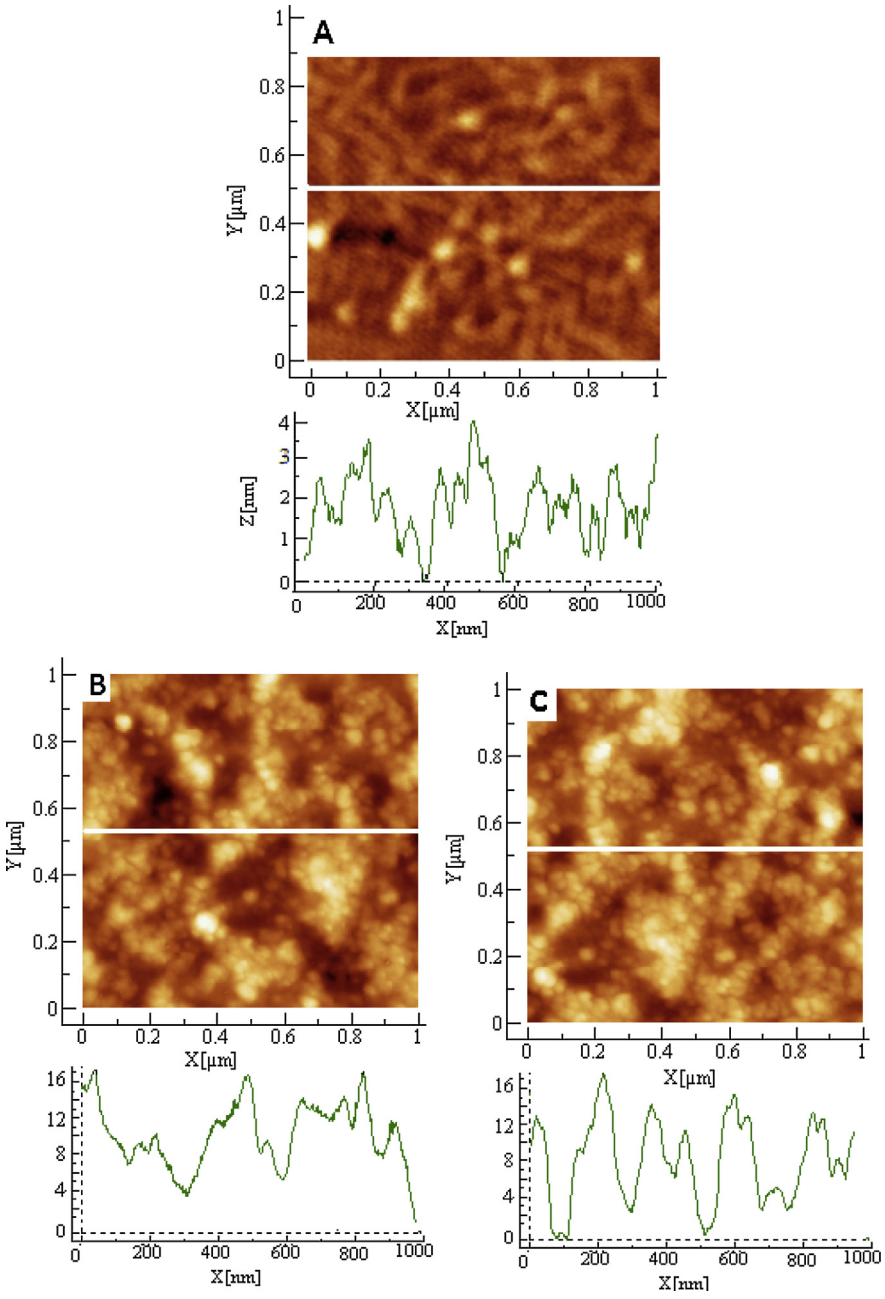

Fig. 4. AFM images of the DNA LbL films prepared at (a) ambient condition (normal pH and room temperature), (b) pH = 13 and (c) temperature = 95 °C.

because after pH 12, the single stranded DNA starts converting into coil. Consequently, the distance between the dye molecules decreases resulting in a slight increase in FRET efficiency. This situation has been explained schematically in the later part of the manuscript.

3.3. Effect of temperature on DNA and FRET

To study the effect of DNA denaturation on the FRET between Acf and RhB, DNA LbL films at different temperature and Acf–RhB mixed LbL films in presence of DNA at different temperature were prepared. DNA monolayer was deposited on a PAH layer at different temperature starting from 30° C to 95° C. The variation of absorbance intensity and FRET efficiency in case of temperature change is very similar to that of pH change. The absorbance intensities of the UV–vis absorption spectra of the corresponding LbL films are shown in Fig. 3(a). It is observed that the absorbance increases remarkably for the films prepared at higher temperature, up to 80 °C (Fig. 3(a). Further increase in temperature after 80 °C results in a remarkable decrease in absorption intensity due to coil formation of the totally denatured DNAs [12]. The coil formation of DNA reduces the length of the transition dipole moment vector leading to a sharp decrease in the absorption intensity. In one of our earlier work on immobilization of denatured single stranded DNA in LbL films [12], we reported that the absorption intensity increases for DNA LbL films prepared up to 80 °C and beyond this temperature the absorption intensity decreases. Fig. 3(b) shows the variation of FRET efficiencies between Acf and RhB in the Acf-RhB mixed LbL films prepared in presence of DNA at different temperature. Here also the trend and nature of FRET efficiency with temperature are almost similar to that of pH change. Up to 60 °C the FRET efficiencies remained unchanged and above this

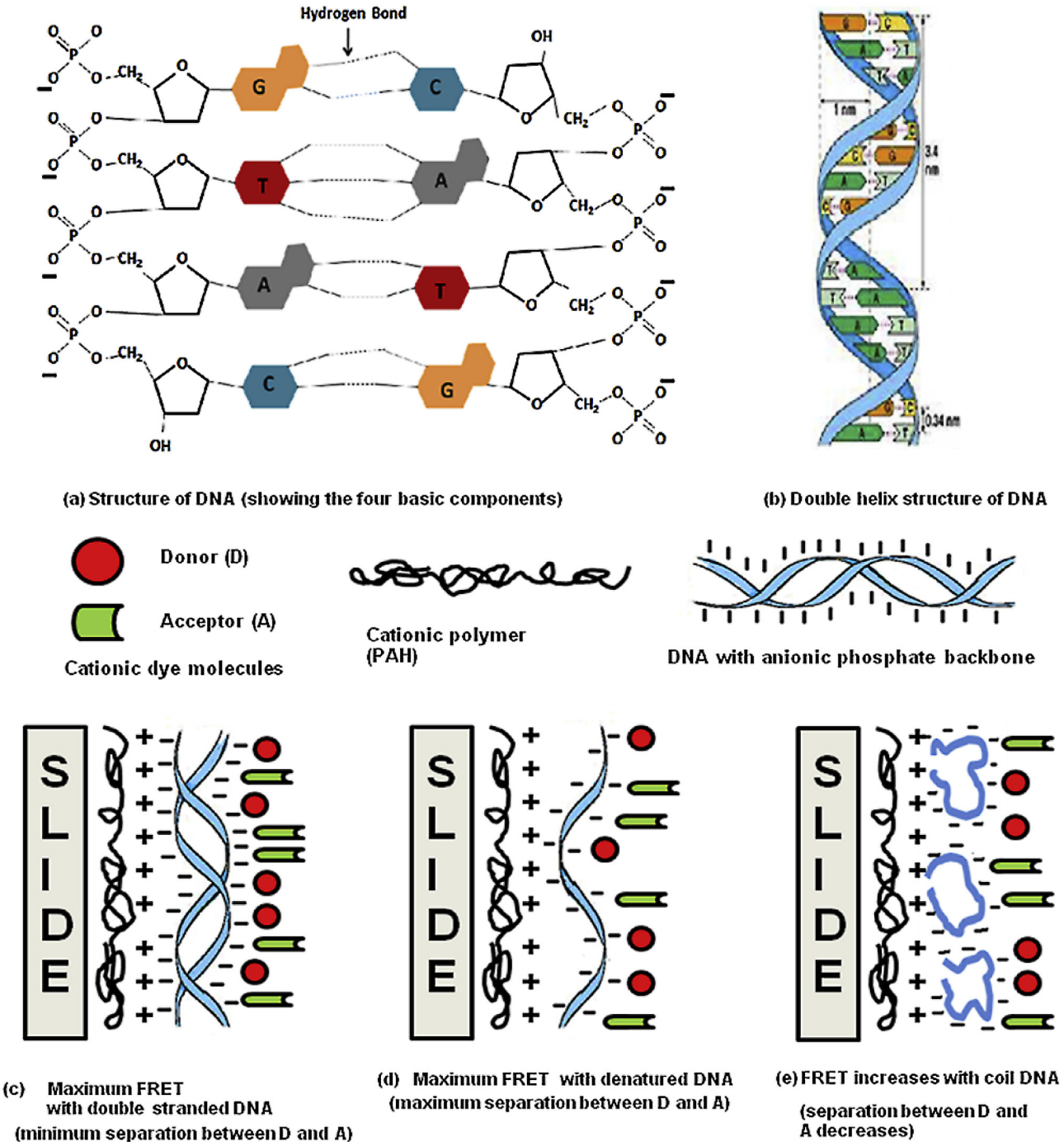

Fig. 5. Schematic diagram shows (a) the four bases of DNA and (b) double helix structure of DNA. Attachment of FRET pair (Acf + RhB) with (c) DNA double helix structure, (d) DNA denatured form and (e) DNA coil form.

temperature the efficiencies started decreasing up to 90 °C. Above 90 °C, slight increase in FRET efficiency was observed. This suggests that with increase in temperature denaturation of DNA occurs and after complete separation of the two strands, formation of coiled single stranded DNA is inevitable (Table 2).

3.4. Atomic force microscopy study of DNA

To check the difference in the surface morphology of the DNA–PAH LbL films before and after melting we have measured the AFM image of the DNA films prepared at different pH and temperature (Fig. 4). Values of RMS roughness, roughness average and average heights as extracted from the AFM images are listed in Table 3. Height profile analyses along with the line marked on the images are also shown.

From the AFM images it has been observed that almost all the substrate surface is completely covered by the DNA molecules in

Table 3
Physical parameters extracted from AFM images.

	Ambient condition	pH = 13	Temp = 95 °C
RMS roughness (nm)	0.5993	4.1591	4.2657
Roughness average (nm)	0.4839	3.3996	3.4933
Average height (nm)	2.9236	12.0051	13.1076

the backbone of PAH in LbL films. Interestingly, AFM images of the LbL films prepared at different temperature and pH values before denaturation (temperature < 80 °C and pH < 11) show almost similar topology. DNA molecules lying flat on the substrate surface are clearly visible. From the height profile analysis it has been observed that height of both the films varies from 0 to 3.5 nm. Again the AFM images of the DNA films prepared after denaturation at pH 13 and temperature 95 °C also reveal identical topology. Here DNA molecules do not remain flat on the films, rather, they posses coiled structure. Height profile analysis show that thickness of the films varies within 0–16 nm.

Therefore, the films prepared before and after denaturation show completely different morphology. Considering the diameter of the DNA molecule ~2–2.5 nm, it seems that the DNA molecules lie flat on the films before denaturation. Again after denaturation the DNA molecules forms coil and possess circular structure. Therefore, AFM investigation gives visual evidence of change in DNA conformation in LbL films before and after denaturation.

3.5. Schematic diagram

The double stranded DNA is composed of two long polynucleotide strands and the complementary bases of the two strands are attached by hydrogen bonds. There are four types of bases in DNA viz., Adenine (A), Thymine (T), Guanine (G) and Cytosine (C), as shown in Fig. 5(a) and (b). The bases lie horizontally between the two spiraling polymer strands. The distance between two consecutive base pairs is 0.34 nm [33–35]. The negatively charged phosphate deoxyribose backbones on either side of the base pair can be labeled with different functional groups or dye molecules [33–35]. A schematic representation showing the attachment of RhB (A) and Acf (D) on the negatively charged phosphate backbone of double stranded DNA is given in Fig. 5(c). Both the dyes Acf and RhB used in the present study are cationic. In presence of DNA, the cationic dyes Acf and RhB attach with the DNA strands through the electrostatic attraction with the negatively charged phosphate backbone of DNA (Fig. 5(c)). Before denaturation, the FRET efficiency between the dyes adsorbed on to DNA remains almost constant. This is because the distance between them is almost constant. Again after denaturation (at high pH and temperature) the FRET efficiency decreases. At this stage the DNA strands get separated and remain as single stranded that increases the distance between the dyes. This has been shown schematically in Fig. 5(d). However, above pH 12 and temperature 90° C the FRET efficiency again increases slightly. During this stage the single stranded DNA forms coiled structure and thus the distance between the dyes is lessened to a slight extent. This situation has been shown in Fig. 5(e).

4. Conclusion

The experimental data suggest that the presence of DNA increases the FRET efficiency between two laser dyes Acf and RhB in LbL film. Increase in pH or temperature of DNA results in denaturation followed by coil formation of the DNA. The denaturation of DNA leading to increase in the intermolecular separation between the Acf and RhB molecules eventually results in a decrease in FRET efficiency. On the other hand, the coil formation of the DNA strands marginally decreases the intermolecular distance between Acf and RhB molecules resulting in corresponding increase in FRET efficiency. Taken together, it can be concluded that by observing the change in FRET efficiency between two laser dyes attached to DNA strands, it would be possible to detect the altered DNA conformation in the changed environment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2014.08.029.

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