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Binding Interaction of Cationic Phenazinium Dyes with Calf Thymus DNA: A Comparative Study

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Absorption, steady-state fluorescence, steady-state fluorescence anisotropy, and intrinsic and induced circular dichroism (CD) have been exploited to explore the binding of calf thymus DNA (ctDNA) with three cationic phenazinium dyes, viz., phenosafranin (PSF), safranin-T (ST), and safranin-O (SO). The absorption and fluorescence spectra of all the three dyes reflect significant modifications upon interaction with the DNA. A comparative study of the dyes with respect to modification of fluorescence and fluorescence anisotropy upon binding, effect of urea, iodide-induced fluorescence quenching, and CD measurements reveal that the dyes bind to the ctDNA principally in an intercalative fashion. The effect of ionic strength indicates that electrostatic attraction between the cationic dyes and ctDNA is also an important component of the dye−DNA interaction. Intrinsic and induced CD studies help to assess the structural effects of dyes binding to DNA and confirm the intercalative mode of binding as suggested by fluorescence and other studies. Finally it is proposed that dyes with bulkier substitutions are intercalated into the DNA to a lesser extent.

1. Introduction

Studies on the supramolecular interactions of drugs and organic dyes with various biological targets are gaining increasing importance for the perception of structural and functional features of biomacromolecules so as to simulate the biophysical processes.[1] Characterization of the interaction of small molecules with DNA provides valuable information for the development of effective therapeutic agents that helps to control gene expression.[2] Small molecules serve as analogues in studies of protein−nucleic acid recognition, as sensitive probes for monitoring nucleic acid structure, provide site-specific affinity for molecular biology and yield rationales for drug design.[3] Investigations reveal that binding affinities and sequence specificity of small molecules toward the biomacromolecules are controlled by various structural and electronic factors.[4] These studies lead to the design of new and effective drugs against various deadly diseases.[2] Miniaturization of biosensors and biochips and fabrication of nanometric objects using a DNA template and DNA machines are some of the emerging fascinating applications of present day DNA research.[5]

Calf thymus DNA (ctDNA) is a polymer. The DNA backbone contains an alternating sugar phosphate sequence. ctDNA has a relatively low protein content with a highly polymerized skeleton. Interaction of DNA with drug molecules, in general, involve three types of binding modes: (i) electrostatic binding between the negatively charged DNA phosphate backbone that is along the external DNA double helix and the cationic or positive end of the polar drug, (ii) groove binding involving hydrogen bonding or van der Waals interaction with the nucleic acid bases in the deep major groove or the shallow minor groove of the DNA helix and finally, (iii) intercalative binding where the drug intercalates itself within the nucleic acid base pairs.[6,7] Among the three modes, intercalative binding is the most effective for drugs targeted to DNA.[8] For investigating such binding characteristics of organic dyes to DNA, sensitive spectral techniques have been successfully exploited along with various other techniques.[8]

Phenazinium dyes, namely, phenosafranin (PSF, 3,7-diamino-5-phenyl phenazinium chloride), Safranin-T (ST, 3,6-diamino-2,7-dimethyl-5-phenyl phenazinium chloride), and Safranin-O (SO, 3,7-diamino-2,8-dimethyl-5-phenyl phenazinium chloride) have extensively been used in semiconductors, as energy sensitizers, and as probes for studying various microheterogeneous environments including micelles, reverse micelles and polymeric matrices.[9–12] All three dyes are red colored with a planar tricyclic phenazinium moiety and bear a positive charge. PSF, ST, and SO are water-soluble dyes with the corresponding broad absorption peaks in the visible region.[9] It is known that PSF and ST interact with DNA molecules, and because of the presence of a planar phenazine ring, intercalative mode of binding within the relatively nonpolar interior of DNA helix has been projected as a promising proposition.[8,13] Cationic charge on the central unit is likely to help in improving the binding affinity due to the electrostatic attraction between the probe and the DNA phosphates. In the present paper, we report a comparative study of the binding interaction of ctDNA with PSF, ST, and SO; the probes differing systematically in the bulkiness of the substitution (Scheme 1). Absorbance, fluorescence, fluorescence anisotropy, and circular dichroism (CD) studies infer that all the three dyes bind to the DNA mainly through intercalation, the extent of which follows the order PSF > ST > SO because of the gradual increase in the steric bulk introduced on the phenazinium moiety. There remains, however, a contribution from the electrostatic binding as well.

2. Materials and Methods

PSF, SO, and ctDNA were purchased from Sigma-Aldrich (USA). ST was purchased from Fluka (USA). All the dyes and

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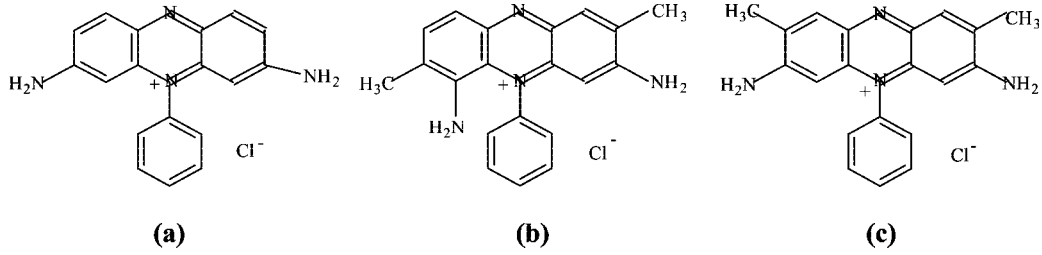
SCHEME 1: Structures of (a) PSF, (b) ST, and (c) SO



ctDNA were used as received without further purification. *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid] (HEPES) buffer, urea, and KI were obtained from SRL (India) and were used as received. Stock solutions were prepared by dissolving the solid DNA in HEPES buffer (pH = 7) and stored at 4 °C. Freshly prepared DNA solution was used for the experiments. The purity of DNA was verified by monitoring the ratio of absorbance at 260 nm to that at 280 nm, which was in the range 1.8−1.9. The concentration of DNA was determined spectrophotometrically, using $\epsilon_{DNA} = 13600\ M^{-1}\ cm^{-1}$ at 258 nm.[14]

Absorption and steady-state fluorescence measurements were carried out using a Shimadzu MPS 2000 spectrophotometer and a Spex Fluorolog-2 spectrofluorimeter equipped with DM3000F software, respectively. Steady-state fluorescence anisotropy measurements were performed with a Perkin-Elmer spectrofluorimeter (model LS55). The steady-state anisotropy, *r*, was defined by

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

where I_{VV} and I_{VH} are the emission intensities obtained with the excitation polarizer oriented vertically and emission polarizer oriented vertically and horizontally, respectively. The *G* factor was defined as

$$G = \frac{I_{HV}}{I_{HH}} \quad (2)$$

where the intensities I_{HV} and I_{HH} now refer to the vertical and horizontal positions of the emission polarizer, with the excitation polarizer being horizontal. CD spectra were recorded on a JASCO J-720 spectropolarimeter, using a rectangular quartz cuvette of path length 1 cm. Spectra shown are averages of four successive scans recorded at a scan speed of 50 nm/min, from which the appropriate blanks have been subtracted and the data were subjected to noise reduction analysis. All the experiments were performed at ambient temperature (300 K) with air-equilibrated solutions. Throughout the experiment, pH of the medium was kept constant at 7 using HEPES buffer.

3. Results and Discussions

3.1. Absorption Study. Absorption spectra of aqueous solutions of PSF, ST, and SO show quite similar broad, unstructured lowest-energy bands with maxima at around 520 nm.[15,16] Figure 1 shows the absorption spectra of the dyes in HEPES buffer at pH 7 in the presence of various concentrations of ctDNA. The concentrations of all the dyes were maintained at 3 μM to make an easier comprehension and comparison of the extent of binding interaction of the different dyes to DNA. Dye concentrations in all the cases were measured from their respective ϵ values.[15]

Addition of the DNA leads to a remarkable change in the absorption spectra of the dyes. The changes occur in the form of decrease in absorbance and appreciable red shift of the absorption maxima, the shift being 17, 15, and 18 nm for PSF, ST, and SO, respectively, at the respective saturation level of interaction. Such type of bathochromism in the dye−DNA binding study suggests strong interaction between the DNA and the chromophore.[8,14,17] The DNA-induced spectral changes in Figure 1 can be explained in terms of a change of local polarity around the fluorophore (dye), which in turn affects the stabilization of its different energy levels. With decrease in the local polarity in ctDNA environment compared to pure buffer solution, the energy gap between the highest-occupied molecular orbital (HOMO) and the lowest-unoccupied molecular orbital (LUMO) of the dyes reduces to give rise to the observed bathochromism (Scheme 2). There is no isosbestic point observed for any of the three dyes. This implies that 1:1 dye:DNA stoichiometry is not maintained during the binding process and/or there is more than one type of binding. This prevented determination of the binding constant from the absorption studies.

3.2. Steady-State Fluorescence Study. The fluorescence spectra of aqueous solutions of PSF, ST, and SO show quite alike broad, unstructured bands with maxima at around 585 nm.[15] Addition of DNA leads to quenching of the dye

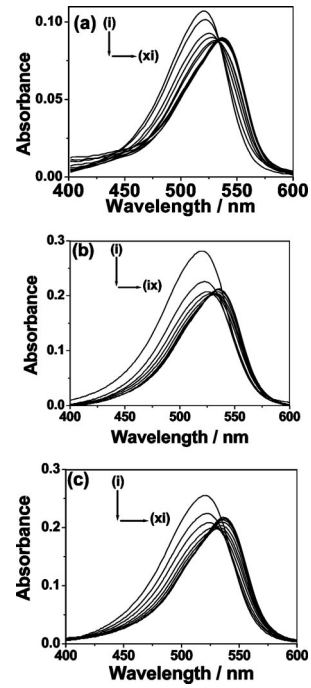
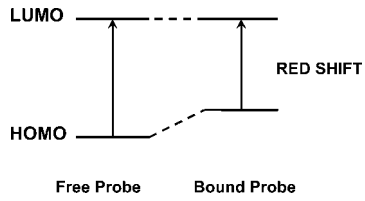


Figure 1. Absorption spectra of (a) PSF, (b) ST, and (c) SO in the presence of different ctDNA concentrations. Curves corresponds to (a) 0, 5, 15, 20, 40, 50, 80, 100, 120, 130, and 140 μM of DNA; (b) 0, 9, 18, 37, 46, 60, 75, 90, and 102 μM of DNA; (c) 0, 5, 19, 33, 61, 97, 126, 174, 199, 225, and 246 μM of DNA, respectively. Concentrations of all the dyes were 3.0 μM.

SCHEME 2: Energy Level Diagram for the Electronic Transition (Absorption) of the Dyes upon Binding with ctDNA



fluorescence along with a slight blue shift (~7 nm). The extent of quenching, however, was different for the different dyes (Figure 2). For the same concentration of added DNA, the quenching of fluorescence was in the order PSF > ST > SO. As a consequence, the concentration of DNA required for the saturation of the fluorescence intensities was found to be in the order SO > ST > PSF (insets of Figure 2).

The observed fluorescence quenching reveals a binding interaction between the probe and the DNA. As already mentioned, the quenching of fluorescence of the dyes through DNA binding is in the order: PSF > ST > SO. The ratio of peak fluorescence intensity in the presence and in the absence of ctDNA (F/F_0) has been plotted as a function of DNA

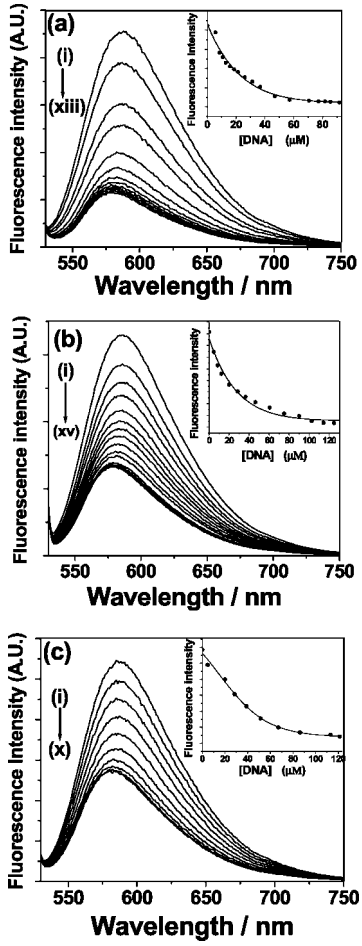


Figure 2. Emission spectra of (a) PSF, (b) ST, and (c) SO in the presence of different ctDNA concentrations. (λ_{exc} = 520 nm). Curves correspond to (a) 0, 6, 9, 13, 21, 37, 47, 71, and 92 μM of DNA; (b) 0, 4.6, 8.5, 12, 20, 36.5, 46, 60.5, 75, 90, 102.5, and 114 μM of DNA; (c) 0, 5, 20, 28.5, 39, 51.5, 67, 86, 113, and 122 μM of DNA, respectively. Insets show the variation of the respective fluorescence intensities with DNA concentrations.

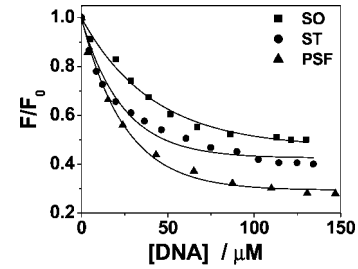


Figure 3. Relative extent of fluorescence quenching for PSF, ST, and SO as a function of ctDNA concentration.

concentration for all the three dyes (Figure 3). The plot indicates that the degree of binding decreases with increasing steric bulk of the phenazinium part of the dyes, i.e., the binding interaction increases gradually from SO to PSF through ST.

3.3. Effect of Urea on the DNA Binding. Urea induced denaturation studies through steady-state fluorescence measurements have been exploited to detect the changes in tertiary structure and conformational stability of globular proteins[18] and DNA. Urea is a well-known denaturant that destabilizes the double stranded DNA helix.[19–23] Denaturation of the biomacromolecules often results in the release of entrapped probe molecules, leading to modification in the fluorescence behavior of the probe.[24] In the present study, we have used this skill to collect evidence in support of the proposed intercalative mode of binding and to reveal the extent of intercalation for the three dye systems in ctDNA. Gradual addition of urea to the dye−DNA complex modifies the fluorescence spectra of the dyes in a manner qualitatively opposite to that observed in the case of gradual addition of ctDNA to the dye solutions. The fluorescence intensity shows progressive enhancement with increase of the added urea concentration, along with a red shift of about ~7 nm in each case, indicating the release of the dye molecules from the DNA environment to the buffer solution upon addition of urea. In fact, the fluorescence intensity of the DNA-bound dyes reach that of the free dye at high enough concentration (6−10 M) of urea. This suggests that urea is able to completely liberate the probes from within the DNA strands.

The relative extent of intercalation of each dye as a function of urea concentration, given by the ratio of its fluorescence intensities in the presence and in the absence of urea (F/F_0), is shown in Figure 4. It is observed that the concentration of urea required for complete ejection of the DNA-bound dye molecules into the bulk aqueous buffer phase follows the order PSF > ST > SO. The observation suggests that the extent of binding of the dyes also follows the same order, i.e., PSF is intercalated into the DNA to the greatest extent and SO to the least extent, with ST in between. As mentioned above, this order is in conformity with the size of the steric substitutions on the phenazinium part of the dyes.

3.4. Fluorescence Quenching Study. To further elucidate the mode of binding of the dyes with DNA, their fluorescence quenching in the ctDNA environment was studied using potassium iodide as a quencher and the correlation between the degree of accessibility of each probe molecule to the quencher and its steric bulk was examined.[18] As is known, intercalation of small molecules into the DNA double strands protects the entrapped molecules from an ionic quencher.[17,25,26] Electrostatic binding and groove binding, on the contrary, leave the probe molecule exposed to the bulk aqueous phase and does not appreciably obstruct the approach of the quencher to it.[25] Moreover, groove binding is likely to have no differential effect on the three dyes in terms of their steric bulk. Intercalation,

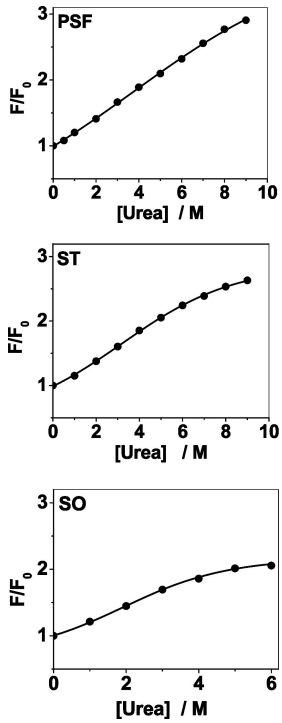


Figure 4. Variation of fluorescence intensity of ctDNA-bound PSF, ST, and SO as a function of urea concentration.

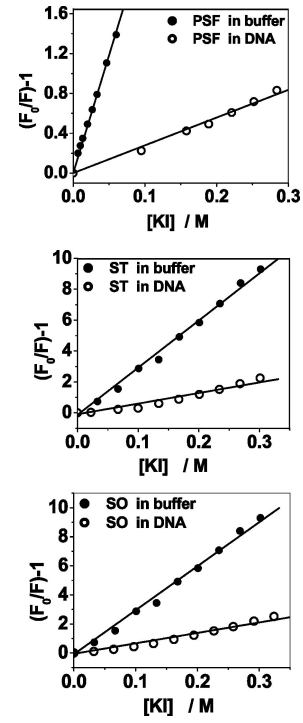


Figure 5. Stern−Volmer plots for fluorescence quenching of PSF, ST, and SO by KI in aqueous and ctDNA environments.

however, provides more protection for the less bulky fluorophore and the approach of the ionic quencher toward the fluorophore is restricted.

Thus, intercalative binding of a fluorophore should lead to reduction in the extent of its fluorescence quenching in ctDNA environment in comparison to that in buffer solution.[27] For groove binding, on the other hand, the effective quenching efficiency of a quencher like KI toward the fluorophore in ctDNA environment is higher than that for the free probe.[28] This is because the addition of salt or a strong electrolyte (such as KI) to the solution releases the DNA-bound dye cations from the groove and causes a decrease in the fluorescence yield. Therefore, for groove binding of the dye to DNA, fluorescence quenching with anionic quenchers will be enhanced appreciably, in contrast to the expected protection of the probe fluorophores by the DNA helix. Based on these ideas, fluorescence quenching of the dyes by KI was studied following the Stern−Volmer equation

$$F_0/F = 1 + K_{SV}[Q] \quad (3)$$

where F_0 and F are the fluorescence intensities in the absence and in the presence of the quencher KI (Q) and K_{SV} is the Stern−Volmer quenching constant. K_{SV} is indicative of the accessibility of the bulky quencher (iodide) to the fluorophore. The slopes of the ($F_0/F - 1$) vs [KI] plots yield the values of K_{sv} (Figure 5), which are shown in Table 1 for all the three probes.

The results from Figure 5 and Table 1 show that K_{SV} values for the dyes in the presence of DNA are much lower than those in the absence of it, confirming intercalative binding of the dyes within ctDNA base pairs. Again, it is observed that the relative reduction of K_{SV} in the presence of DNA is highest for PSF and least for SO. The observation can be rationalized in light of the relative bulk of the probes, due to which their intercalation with DNA is restricted most in the case of SO and least for PSF. This causes the former to be the most accessible to the quencher and the latter to be the least.

TABLE 1: Comparison of K_{SV} Values for the Three Dyes in Buffer and ctDNA Environments

fluorophore	K_{SV} in buffer	K_{SV} in drug−DNA complex	relative reduction in K_{SV} (%)
PSF	23.7	2.9	88
ST	30.5	6.4	79
SO	31.6	7.2	77

3.5. Effect of Ionic Strength. Since each of the chosen fluorophores carries a positive charge and DNA has a negative polyphosphate backbone, the effect of ionic strength on dye−DNA binding has been examined by studying the binding in the presence of the strong electrolyte (NaCl) to verify if there is significant electrostatic interaction between the dyes and DNA.[8,17] Increased ionic strength screens the phosphate−phosphate repulsion prompting the helix to shrink due to a decrease in the unwinding tendency caused by electrostatic repulsion between the negatively charged phosphate groups.[14] Thus an increase in ionic strength leads to weakening of electrostatic attraction between the positively charged fluorophore molecule and DNA. Figure 6 shows the relative fluorescence increment of the dye−ctDNA complexes in the presence of NaCl.

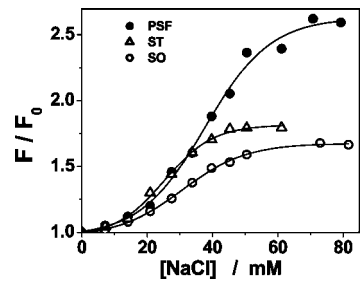


Figure 6. Variation of fluorescence intensity of ctDNA-bound PSF, ST, and SO as a function of NaCl concentration.

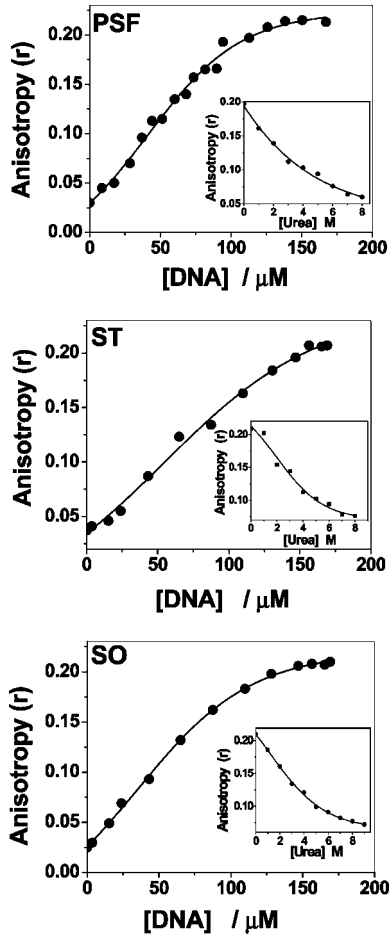


Figure 7. Variation of fluorescence anisotropy as a function of increasing concentration of ctDNA for PSF, ST, and SO. Insets show variation in the fluorescence anisotropy as a function of increasing concentration of urea for the DNA-bound dyes.

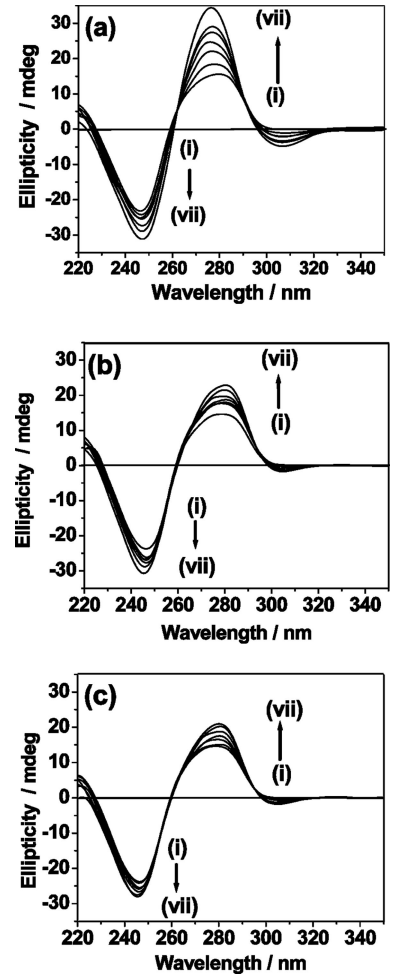


Figure 8. Intrinsic CD spectra of ctDNA with varying concentrations of (a) PSF (curves i−vii) represents PSF concentrations of 0, 3, 6, 9, 12, 15, and 21 μM, respectively), (b) ST (curves i−vii represents ST concentrations of 0, 3, 6, 12, 18, 24 and 27 μM, respectively), and (c) SO (curves i−vii represents SO concentrations of 0, 3, 6, 9, 15, 21, and 30 μM, respectively). DNA concentration in each case was 62 μM.

In all the cases, fluorescence intensity of the probes increases upon addition of NaCl up to the saturation level of their interaction. Figure 6 thus implies that electrostatic interaction also plays a role in the probe−DNA binding process. Involvement of both intercalative and electrostatic binding interactions supports the nonexistence of the isosbestic points in the absorption study (*vide* section 3.1). Figure 6 further reveals that the relative increase in the fluorescence of the DNA-bound dyes as a function of NaCl concentration follows the order: PSF > ST > SO, indicating that addition of NaCl affects the DNA-binding interaction of PSF the most and that of SO the least.

3.6. Steady-State Fluorescence Anisotropy Study. Measurement of fluorescence anisotropy provides significant information about the physical characteristics and the nature of the environment of biological probes. Any factor affecting size, shape, and segmental flexibility of a molecule affects the observed anisotropy.[18] Increase in the rigidity of the environment surrounding the fluorophore results in an increase in the fluorescence anisotropy. Monitoring the anisotropy can thus help in finding the probable location of a probe in microheterogenous environments like proteins, DNA, micelles, reverse micelles, and cyclodextrins.[29–31] Figure 7 shows the variation of fluorescence anisotropy with increasing concentration of ctDNA for the three fluorophores PSF, ST, and SO.

The plots show a marked increase in the fluorescence anisotropy on increasing the concentration of ctDNA in all the three cases suggesting that the fluorophores are trapped in a motionally restricted region within ctDNA compared to that in pure buffer solution. In all three dye systems, at the completion of the dye−DNA interaction, steady-state anisotropy (r) values are determined to be almost the same (~0.2). Upon addition of urea, the reverse trend, i.e., a steady decrease in the fluorescence anisotropy, is observed in all the three cases (insets of Figure 7). This implies that the motional restriction imposed by the DNA environment on the fluorophores is relaxed upon addition of urea, due to the effect of denaturation as discussed in section 3.3. Figure 7 shows that the saturation value of steady-state anisotropy (r) at the completion of dye−ctDNA interaction comes out to be almost the same (~0.2) for all the three dyes, apparently indicating that these measurements are unable to sort their relative extent of intercalation within the ctDNA. The reason behind this might be that irrespective of the extent of intercalation, the overall tumbling motion of the dye−DNA complex, which is almost similar in dimension in all the three cases, is predominantly responsible for determining the fluorescence anisotropy.

3.7. CD Study. Binding of a dye with DNA may stabilize or destabilize the latter. The conformational aspects of dye−DNA interaction, including changes in the secondary and tertiary structures of DNA, were studied using both intrinsic and induced CD spectroscopy. Literature reports suggest that the secondary structures of DNAs are perturbed by intercalation with small

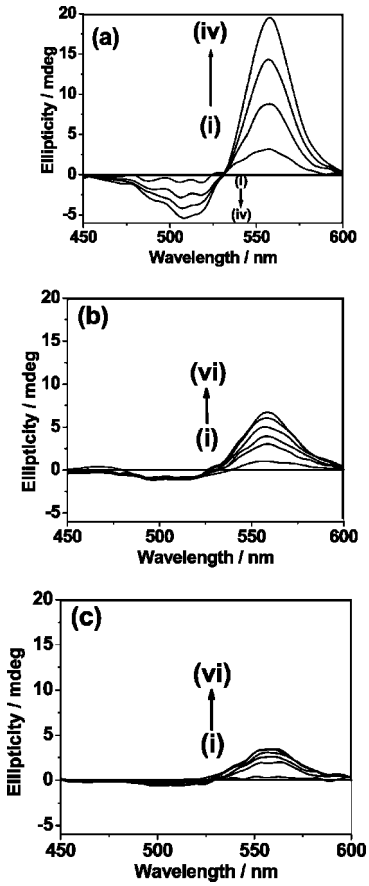


Figure 9. Induced CD spectra of 20 μM of (a) PSF, (b) ST, and (c) SO titrated with increasing concentrations of ctDNA Curves i−vi represent DNA concentrations of 8.4, 23.6, 38.8, 61.7, 89.5, and 120.6 μM, respectively.

molecules.[32,33] Binding of small molecules with DNA may lead to drive the entire assembly of the multistranded DNA structure. Intercalation induced perturbation of the DNA secondary structure has been traditionally demonstrated by a shift in the melting temperature of the DNA strands.[33] In the present study, however, we have exploited circular dichroism to identify the perturbation in the secondary structure of ctDNA due to intercalation of the three phenazinium dyes. The backbone conformation of ctDNA shows a CD spectrum characteristic of the right-handed B form in the far-UV region (220−320 nm). Structural alterations of the DNA caused by its interaction with probes are reflected in changes in this intrinsic CD spectrum.[34] Figure 8 shows the CD spectrum of free DNA in HEPES buffer at pH 7, having a positive peak at ~277 nm and a negative peak at ~247 nm. These bands are caused by stacking interactions between the bases and the helical suprastructure of the polynucleotide that provide an asymmetric environment for the bases.[35] The spectra are characteristic of the B form with 10.4 base pairs per turn, which is the normal conformation of DNA in aqueous solution at moderate salt concentration.[36] Gradual addition of each of the three dyes to the DNA solution leads to remarkable perturbations of both the positive and the negative bands (Figure 8). The band intensities are enhanced without appreciable shift in their positions. A plausible explanation of the change in the 280 nm band is the disruption of stacking contacts of the bases, which is required to optimize the binding interactions.[37] These changes may thus reflect local untwisting of the DNA helical backbone and changes in relative orientation of the bases to accommodate the intercalating drug within a particular base pair.[35,37] The extent of increase in the ellipticity is different for the three dyes, being maximum for PSF and minimum for SO, which implies that binding of PSF causes the largest perturbation to the DNA strands and the most extensive destacking and destabilization of the DNA helix. The result can again be rationalized in light of the relative steric bulk on the probes, due to which intercalation to the DNA is restricted most for SO and least for PSF.

Further evidence in favor of the proposed mode of binding of the dyes to DNA was obtained by measuring the visible CD spectra (450−600 nm) induced by interaction with ctDNA. These induced CD spectra occur in a wavelength region where the DNA has no absorption band and thus exclusively monitor changes in the environment of the dye molecules.[37] It is pertinent to note here that the dyes being achiral and planar are not CD active by themselves. Therefore the induced CD spectrum is generated exclusively from the asymmetric arrangement of the dye that has been intercalated into the DNA base pairs.[14] The induced CD spectra for all three DNA-bound dyes showed a strong positive band peaking at 558 nm and a weak negative band at 510 nm (Figure 9). The ellipticity of the positive peak was significantly enhanced as the concentration of DNA was increased. Figure 9 not only confirms the intercalative mode of binding of the dyes with DNA but also provides an insight into the specific mode of intercalation. Indeed, the present findings permit one to distinguish between the possible arrangements of the dye within the intercalation site. The dye molecule can have its long axis oriented either parallel (characterized by negatively induced CD spectra) or perpendicular (characterized by positively induced CD spectra) to the long axis of the DNA base pair. Accordingly, the positive signal of the induced CD spectrum in Figure 9 indicates that the dyes intercalate with their long axes perpendicular to that of the DNA base pairs.[37,38] An inspection of the induced CD spectra obtained for the three dyes reveals that the ellipticity is increased in the order PSF > ST > SO. It can therefore be inferred that PSF, being sterically smaller than the other two can penetrate to the greatest extent into the helices of DNA leading to maximum asymmetry in the arrangement within the ctDNA.

Conclusion

The present work reports a study of the interaction of three analogous biological photosensitizers, PSF, ST, and SO, with ctDNA. The photophysical behaviors of the dyes are modified remarkably upon binding to the DNA as compared to the buffered aqueous phase. A series of studies involving absorption, steady-state fluorescence, fluorescence anisotropy, CD, and effects of urea and ionic strength indicate that binding of these phenazinium drugs to DNA occurs principally in an intercalative mode. The extent of intercalation follows the order PSF > ST > SO, which is in the reverse order of the steric bulk attached to the planar phenazinium part of the drugs.

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