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Toxic interaction of thionine to deoxyribonucleic acids: Elucidation of the sequence specificity of binding with polynucleotides

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ABSTRACT

The sequence specificity of the intercalative DNA damage of the phenothiazine dye thionine has been investigated by absorbance, fluorescence, circular dichroism and viscosity studies using four synthetic polynucleotides, poly(dA-dT)·poly(dA-dT), poly(dA)·poly(dT), poly(dG-dC)·poly(dG-dC) and poly(dG)·poly(dC). Strong hypochromic-bathochromic effects in absorbance and quenching in fluorescence were observed that showed strong binding of thionine to these polynucleotides. Scatchard plots revealed non-cooperative binding and analysis by McGhee–von Hippel equation provided the affinity values in the order of 10^5 M^{-1}. The binding clearly revealed the high preference of thionine to the alternating GC sequences followed by the homo GC sequences. The AT polynucleotides had lower binding affinities but the alternating AT sequences had higher affinity compared to the homo stretches. The results of ferrocyanide quenching studies in fluorescence and viscosity experiments conclusively proved the intercalation of thionine while circular dichroic studies provided evidence for the structural perturbations associated with the sequence specific intercalative binding. The sequence specificity of the intercalative damage of thionine to deoxyribonucleic acid is advanced from this study.

1. Introduction

Sequence specificity of the binding of aromatic molecules with DNA and damage of the B-DNA structural features due to intercalation have been an active topic of research that has remarkable relevance and implications in several biological processes including cancer chemotherapy [1–7]. Strong and highly specific intercalative binding of small molecules with the genomic DNA and consequent structural damages may be effective in preventing further information retrieval from DNA leading to arrest of cell division. Additionally, photosensitization may cause direct or indirect damage to DNA and the cells may also occur in such binding reactions [8,9]. Such photophysical behaviour linked to DNA binding may be exploited for cleavage and damage of the DNA backbone for photodynamic therapy of tumors and other diseases [10,11]. Due to these reasons elucidation of the molecular aspects of the intercalative interaction of aromatic molecules with DNA has been the subject of investigation since the discovery of DNA structure [1–10]. The intercalative binding properties of such molecules can also be harnessed as diagnostic probes for DNA structure and DNA directed therapeutics [1,6,7,12]. Nevertheless, the studies so far have remained inconclusive in terms of a clear understanding the nature, specificity and energetics of the interaction. Consequently the development of effective chemotherapeutic agents remains, to a large extent, an elusive goal and highly challenging [4]. It appears that more concerted effort is required to understand the molecular aspects of the interaction, toxic effects of binding reaction, DNA damage on binding as a function of base sequence and selectivity.

Thionine (3,7-diamino-5-phenothiazinium) (Fig. 1), a positively charged tricyclic heteroaromatic molecule, has been studied for its photoinduced mutagenic actions [13,14], toxic effects, damage on binding to DNA [15], photoinduced inactivation of viruses [16] and use as an impedance based DNA biosensor [16–19]. There have been several studies on the toxicity of thionine and many related phenothiazine dyes [20–25]. Thionine has been shown to inactivate frog sperm nucleus [20], produce toxic effects in anaerobic glycolysis [21], induce structural changes in rat mast cells and block mast cell damage by inhibiting cell metabolism [22]. Very recently, the unique ability of thionine to immobilize proteins and DNA and act as molecular adhesive has been reported [26]. Tuite and Kelly [14], employing spectroscopic techniques, first suggested that thionine binds to DNA by both intercalation as well as outside binding modes. They also proposed that a guanine-cytosine (GC) specificity of thionine binding was not very marked. Using satellite hole spectroscopy, Chang et al. [27] proposed a GC specific binding for thionine. Very recently, from a variety of biophysical and calorimetric studies with three natural DNAs of varying base compositions, we confirmed the intercalative binding and high affinity of thionine to GC rich DNAs [28]. Thus, while

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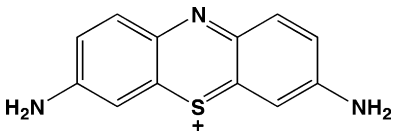

Fig. 1. Chemical structure of thionine.

the mode of binding and the overall base preference of thionine is clear now from the structural and thermodynamic data, the base sequence specificity of binding to DNA is not yet delineated. Towards this goal, we characterized the sequence specificity of thionine interaction through spectroscopic and competition dialysis studies with four sequence specific synthetic polynucleotides poly(dG-dC)·poly(dG-dC), poly(dG)·poly(dC), (dA-dT)·poly(dA-dT), and poly(dA)·poly(dT).

2. Experimental

2.1. Chemicals and apparatus

DNA polynucleotides, poly(dG-dC)·poly(dG-dC), poly(dG)·poly(dC), (dA-dT)·poly(dA-dT), and poly(dA)·poly(dT) were purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA). They were made to a uniform length of about 280 ± 50 base pairs by sonication in a Labsonic sonicator (B. Braun, Germany) using a needle probe. Thionine acetate (thionine/ligand or dye in general hereafter) was obtained from Sigma–Aldrich and was used as received. All other reagents were of analytical grade. Deionised and triple distilled water was used for the preparation of buffer. The buffer solution was filtered through Millipore membrane filters of 0.45 μm (Millipore India Pvt. Ltd. Bangalore, India).

All absorbance spectra were measured on a Jasco V-660 double beam double monochromator spectrophotometer (Jasco International Company, Tokyo, Japan) studies in 1 cm path length quartz cuvettes using the methodologies previously described [29,30]. Steady state fluorescence measurements were performed on a Shimadzu RF5301PC spectrofluorimeter (Shimadzu Corporation, Kyoto, Japan) in fluorescence free quartz cuvettes of 1 cm path length as previously described [29,31]. A Jasco J815 spectropolarimeter equipped with a Jasco temperature controller (model PFD 425 L/15) was used for all circular dichroic measurements at 20 ± 0.5 °C [28–30]. Cannon–Manning semi micro size 75 capillary viscometer (Cannon Instruments Company, State College, PA, USA) was used for viscosity measurements.

2.2. Preparation of solutions

All experiments were performed in 50 mM sodium cacodylate buffer of pH 7.2 at 20 ± 0.5 °C. Concentration of the polynucleotides were determined spectrophotometrically using molar extinction coefficient values reported in the literature [31]. Thionine is highly soluble in aqueous buffer and its solution was freshly prepared each day and kept protected in the dark until use. A molar extinction coefficient (ε) of 54,200 M^{-1} cm^{-1} at 598 nm was used for estimating the concentration of thionine. Beer's law was obeyed in the concentration range employed in this study.

2.3. Experimental measurements

Initially, titration of a constant concentration of thionine with increasing concentration of polynucleotides was performed to elucidate the absorbance maxima and isosbestic points. Thereafter, absorbance titration of a constant concentration of the polynucleotide was performed with increasing concentration of thionine with continuous stirring throughout the titration. The amount of free and bound thionine was determined as follows. Following each addition of thionine to the polynucleotide solution, from the absorbance at the isosbestic point, the total dye concentration present was calculated as described earlier [29,30]. This quantity was used to calculate the expected absorbance at the wavelength maximum. The difference in the expected absorbance and the observed absorbance was used to calculate the amount of bound drug. The amount of free drug was then determined by difference. The extinction coefficient of the completely bound dye was determined by adding a known quantity of thionine to a large excess of polynucleotide and on the assumption of total binding as described earlier [29,30].

For fluorescence measurements the emission spectra of thionine–DNA complexes were monitored in the region 600–700 nm keeping a constant excitation and emission slit width of 5 nm.

The data obtained from spectrophotometric and spectrofluorimetric titrations were then cast into Scatchard plots of r/C_f versus r where r is the number of moles of thionine bound and C_f is the molar concentration of free thionine. All the Scatchard plots revealed negative slope at low r values characteristic of non-cooperative binding isotherms. Hence, these plots were analyzed by the McGhee–von Hippel equation, for non-cooperative binding [32],

$$\frac{r}{C_f} = K_i(1 - nr)\left[\frac{(1 - nr)}{\{1 - (n - 1)r\}}\right]^{(n-1)} \quad (1)$$

where r is the molar ratio of the bound ligand to the polynucleotide, C_f is the free ligand concentration, K_i is the intrinsic binding constant to an isolated binding site, and n is the number of base pairs excluded by the binding of a single dye molecule. The binding data were analyzed using Origin 7.0 software (Microcal Inc., Northampton, MA, USA) as described previously [29,30,33] to give K_i and n.

Protocols developed by Chaires and co-workers [34,35] were followed generally for performing competition dialysis assay. 500 μL of each of the polynucleotide solution (at identical concentration of 75 μM in base pair units) was kept in separate 0.5 mL Spectra/Por® cellulose ester sterile DispoDialyzer units (Spectrum Laboratories, Inc., CA, USA) and dialyzed against a common 1 μM dialysate solution of thionine for 24 h in the dark. The samples were carefully collected and transferred to microfuge tubes. Dissociation of the thionine–polynucleotide complexes was achieved by adding appropriate volume of a 10% (w/v) of sodium dodecyl sulphate solution to make a final concentration of 1% (w/v). The total concentration of bound dye (C_t) was determined by optical density measurements. The concentration of the free thionine (C_f) was determined by using an aliquot of the dialysate solution. The amount of bound thionine (C_b) was determined by the difference, ($C_b = C_t - C_f$) and the data were plotted as bar graph using Origin 7.0 software. The apparent binding constant (K_{app}) was calculated using the relation described by Chaires [35],

$$K_{app} = \frac{C_b}{C_f(S_{total} - C_b)} \quad (2)$$

Quenching studies were carried out with the anionic quencher potassium ferrocyanide ($K_4[Fe(CN)_6]$). The experiments were done by mixing, in different ratios, two solutions, one containing KCl, and the other containing $K_4[Fe(CN)_6]$, in addition to the normal buffer components, at a fixed total ionic strength. Experiments were performed at a constant P/D (DNA base pair/thionine molar ratio) monitoring fluorescence intensity as a function of changing concentration of the ferrocyanide as described previously [28,29]. The data were plotted as Stern–Volmer plots of relative fluorescence intensity (Fo/F) versus $[Fe(CN)_6]^{4-}$ concentration according to Stern–Volmer equation as previously described [36].

Circular dichroism (CD) spectra were recorded on a Jasco J815 spectropolarimeter. A rectangular strain free quartz cell of 1 cm path length was used. Instrument parameters for CD measurements were, scan rate of 50 nm/min. bandwidth of 1.0 nm, and sensitivity of 100 mdeg. The molar ellipticity values [θ] are expressed in terms of DNA base pair in the wavelength range 200–400 nm and per bound thionine in the 300–700 nm region.

For viscosity measurements the time of flow was measured in triplicate with an electronic stop watch Casio model HS 30W (Casio Computer Company Ltd., Japan) to an accuracy of ±0.01 s. Relative viscosities for DNA in the presence or absence of thionine were calculated from the relation,

$$\frac{\eta'_{sp}}{\eta_{sp}} = \frac{\{(t_{complex} - t_o)/t_o\}}{\{(t_{control} - t_o)/t_o\}} \quad (3)$$

where, η_{sp}' and η_{sp} are the specific viscosity of polynucleotides in presence and absence of the thionine, $t_{complex}$, $t_{control}$ and t_o are the average flow times for the complex, free polynucleotide and buffer, respectively. The relative increase in length, L/L_o, can be obtained from a corresponding increase in relative viscosity using the relation,

$$\frac{L}{L_o} = \left(\frac{\eta}{\eta_o}\right)^{1/3} = 1 + \beta r \quad (4)$$

where L and L_o are the contour length of the polynucleotides in the presence and absence of thionine and η and η_o are the corresponding values of intrinsic viscosity (approximated by the reduced viscosity $\eta = \eta_{sp}/C$ where C is the polynucleotide concentration) and β is the slope when L/L_o is plotted against r [30,33].

3. Results and discussion

3.1. Absorbance titration of thionine with polynucleotides and estimation of binding affinity

The effect of the polynucleotides on the absorbance spectrum of thionine was studied. Thionine has characteristic visible absorption spectrum in the 450–700 nm region with a maximum at 598 nm that is convenient to monitor the interaction. Pronounced hypochromic and bathochromic effects were observed in this spectral region of thionine when mixed with increasing concentrations of the polynucleotide duplexes, revealing strong intermolecular association. Such spectral changes have been usually interpreted to arise from a strong interaction between the π electron cloud of the interacting small molecule and the base pairs presumably due to intercalation [37–39]. Representative absorption spectral changes in thionine in the presence of increasing concentrations of poly(dG-dC)·poly(dG-dC) is presented in Fig. 2A. Similar spectral changes were observed in each system but to different extents. The presence of a sharp isosbestic point (marked by an arrow) enabled the assumption of a two state system consisting of bound and free thionine at any particular wavelength. A summary of the optical properties of thionine in the free and bound state with each polynucleotide is presented in Table 1. Binding data obtained from spectrophotometric titrations as per protocols described in Section 2.3 were cast into the form of Scatchard plot of r/C_f versus r. In Fig. 2B, the Scatchard plots of thionine binding to the four polynucleotides studied are depicted. It can be seen that the binding isotherms have similar pattern with negative slope at low r values which indicate non-cooperative binding. The binding affinity of thionine to poly(dG-dC)·poly(dG-dC), poly(dG)·poly(dC), poly(dA-dT)·poly(dA-dT) and poly(dA)·poly(dT) thus evaluated were $(4.72 \pm 0.20) \times 10^5$, $(3.17 \pm 0.30) \times 10^5$, $(1.72 \pm 0.013) \times 10^5$ and $(1.01 \pm 0.023) \times 10^5$ M^{-1}, respectively. These values along with the numbers of excluded binding sites obtained in each case are depicted in Table 2. These results suggest that the affinity of binding of thionine to the GC polynucleotides was higher than that to the AT polynucleotides. Secondly, the affinity was highest with poly(dG-dC)·poly(dG-dC) suggesting the higher preference of thionine to the alternating GC sequences. It was suggested previously that only one GC base pair was preferred at the intercalation site of thionine from the result of studies with natural DNAs [28]. The GC speci-

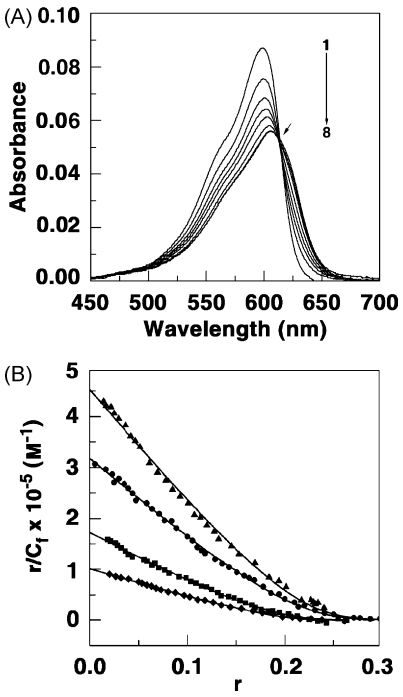

Fig. 2. (A) Absorption spectral changes of thionine (1.6 μmol) treated with 0, 7,14, 21, 28, 35, 42 and 49 μM of poly(dG-dC)·poly(dG-dC) (curves 1–8). (B) Scatchard plots of complexation of thionine with poly(dG-dC)·poly(dG-dC) (▲), poly(dG)·poly(dC) (●), poly(dA-dT)·poly(dA-dT) (■), and poly(dA)·poly(dT) (♦) obtained from spectrophotometric titration data. The solid lines in the plot represent the best fit of the data points to the McGhee-von Hippel equation. All experiments were performed at 20 ± 0.5 °C in 50 mM sodium cacodylate buffer, pH 7.2. Values of K_i (binding constant) and n (number of excluded sites) are presented in Table 2.

Table 1
Summary of the optical properties of free and polynucleotide bound thionine[a].

Property	Thionine (free)	Thionine (bound with)			
		Poly(dG-dC)· poly(dG-dC)	Poly(dG)· poly(dC)	Poly(dA-dT)· poly(dA-dT)	Poly(dA)· poly(dT)
λ_{max} (free)	598	–	–	–	–
ε_f (at λ_{max})	54,200	–	–	–	–
λ_{max} (bound)	–	605	606	609	600
λ_{iso}[b]	–	613	613	613	623
ε_b (at λ_{max})	–	28,760	32,760	29,500	38,000

[a] Units: λ: nm; ε (molar extinction coefficient): M^{-1} cm^{-1}.
[b] Wavelength at the isosbestic point.

Table 2
Binding parameters for the complexation of thionine to polynucleotides evaluated from Scatchard and McGhee–von Hippel analysis of the absorbance and fluorescence titration data[a].

Polynucleotide	Absorbance		Fluorescence	
	$K_i \times 10^{-5}$ (M^{-1})[b]	n	$K_i \times 10^{-5}$ (M^{-1})	n
Poly(dG-dC)· poly(dG-dC)	4.72 ± 0.20	3.18	4.61 ± 0.20	3.15
Poly(dG)· poly(dC)	3.17 ± 0.30	3.36	3.07 ± 0.30	3.41
Poly(dA-dT)· poly(dA-dT)	1.72 ± 0.013	3.76	1.75 ± 0.30	3.74
Poly(dA)·poly(dT)	1.01 ± 0.023	4.17	0.98 ± 0.024	4.09

[a] Average of four determinations.
[b] Binding constants (K_i) and the number of occluded sites (n) refer to solution conditions of 50 mM cacodylate buffer, pH 7.2 at 20 °C. The best-fit parameters of K_i and n were calculated by linear regression analysis and in all the cases the correlation coefficient varied between 0.98 and 0.99.

ficity of thionine observed is similar to that suggested for many classical DNA intercalators like sanguinarine, aristololactam-β-D-glucoside but different from others like daunorubicin that show more complex base specificity [6,13,40].

3.2. Fluorescence titration studies

The fluorescence property of thionine in the 600–700 nm range with maximum at 615 nm when excited at 596 nm was also exploited to understand the DNA interaction phenomena. Binding to the polynucleotides resulted in the quenching of the fluorescence that eventually leads to saturation of the binding sites in each case. A representative fluorescence pattern of the complexation of thionine with poly(dG-dC)·poly(dG-dC) is presented in Fig. 3. Large fluorescence change in each case is indicative of the strong association of molecules to these polynucleotide structures resulting from an effective overlap of the bound molecules with the base pairs. This result also proposes the location of the bound molecules in a hydrophobic environment similar to an intercalated state. The Scatchard binding isotherms revealed non-cooperative binding phenomena (not shown). The binding constants calculated from the fluorescence data using McGhee-von Hippel analysis (*vide paragraph 3 of* Section 2.3) yielded binding constants of $(4.61 \pm 0.20) \times 10^5$, $(3.07 \pm 0.30) \times 10^5$, $(1.75 \pm 0.30) \times 10^5$ and $(0.98 \pm 0.024) \times 10^5\ M^{-1}$ for poly(dG-dC)·poly(dG-dC), poly(dG)·poly(dC), poly(dA-dT)·poly(dA-dT) and poly(dA)·poly(dT) and these values are also depicted in Table 2. It can be seen that these are in excellent agreement with the results obtained from spectrophotometric analysis.

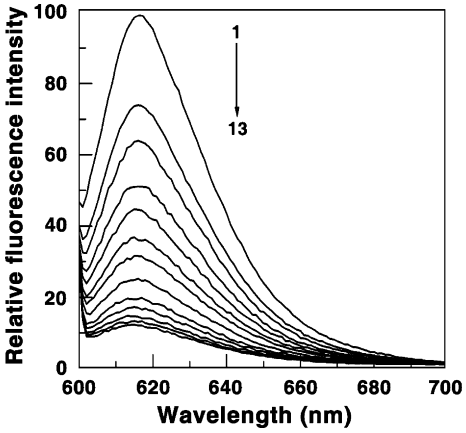

Fig. 3. Fluorescence spectral changes of thionine (5 μM) treated with 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 100 and 120 μM of poly(dG-dC)·poly(dG-dC) (curves 1–13) in 50 mM sodium cacodylate buffer of pH 7.2 at 20 ± 0.5 °C.

3.3. Fluorescence quenching studies and viscosity measurements

The mode of DNA binding of thionine was speculated previously to be intercalative [14,27,41]. We confirmed the intercalative binding through viscometric and fluorescence quenching studies with natural DNAs [28]. Here, we probed the same using fluorescence quenching experiments in presence of $[Fe(CN)_6]^{4-}$. Quenching experiments are straight forward and provide evidence for the location of the bound molecules to be either on the outside or inside of the helix. An anionic quencher would not be able to penetrate the negatively charged barrier around the helix and if the bound thionine molecules are buried within the DNA helix by intercalation little or no change in its fluorescence is expected. Stern–Volmer plots for the quenching of thionine-polynucleotide complexes are shown in Fig. 4A. Results clearly indicate that free thionine molecules are quenched efficiently. Very little quenching was observed in case of complexes with poly(dG-dC)·poly(dG-dC), and poly(dG)·poly(dC). Comparatively more quenching was seen with poly(dA-dT)·poly(dA-dT) and poly(dA)·poly(dT). This result indicated that the thionine molecules bound to the GC polynucleotides are located in a relatively more protected environment, presumably more deeply and/or strongly intercalated compared to that in the AT polynucleotides. The Stern–Volmer quenching constants calculated for free thionine and its complexes with poly(dG-dC)·poly(dG-dC), poly(dG)·poly(dC), poly(dA-dT)·poly(dA-dT) and poly(dA)·poly(dT) were 41, 14, 21, 34 and 38 L/mol, respectively. From these results it can be inferred that the bound thionine molecules are sequestered away from the solvent indicating strong intercalative binding to the GC polymers

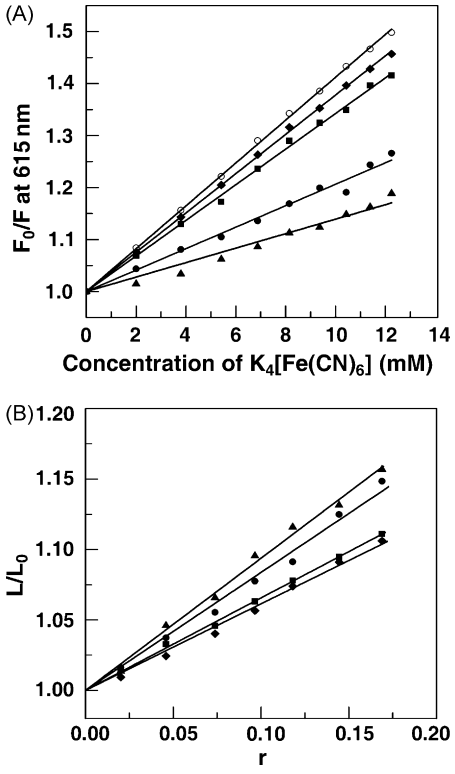

Fig. 4. Stern–Volmer plots for the quenching (upper panel) of thionine (○) and its complexes with poly(dG-dC)·poly(dG-dC) (▲), poly(dG)·poly(dC) (●), poly(dA-dT)·poly(dA-dT) (■), and poly(dA)·poly(dT) (♦) with increasing concentration of $[Fe(CN)_6]^{4-}$ ion in 50 mM sodium cacodylate buffer, pH 7.2 at 20 ± 0.5 °C. Concentration of the dye, polynucleotide and K^+ ion were kept constant. (Lower panel) A plot of increase in helix contour length (L/L_o) versus r for the complexation of poly(dG-dC)·poly(dG-dC) (▲), poly(dG)·poly(dC) (●), poly(dA-dT)·poly(dA-dT) (■), and poly(dA)·poly(dT) (♦), with thionine in 50 mM sodium cacodylate buffer, pH 7.2 at 20 ± 0.5 °C. All the linear fits had linear coefficients >0.96.

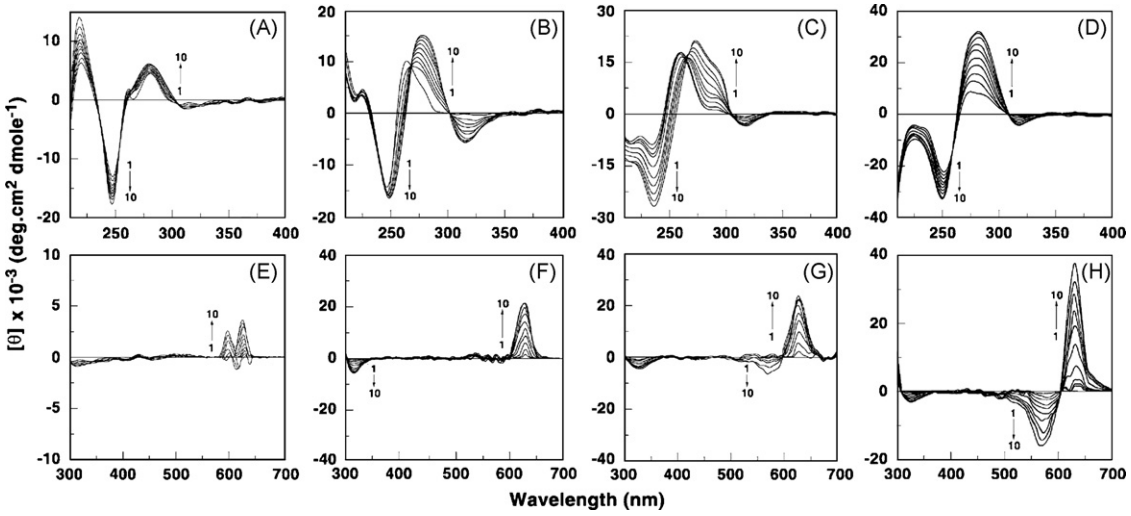

Fig. 5. Representative intrinsic circular dichroic spectra (upper panels) 30 μM of (A) poly(dA)·poly(dT) (B) poly(dA-dT)·poly(dA-dT) (C) poly(dG)·poly(dC) and (D) of poly(dG-dC)·poly(dG-dC) treated with 0.0, 1.5, 3.0, 6.0, 12.0, 15.0, 18.0, 21.0, 27.0, 30.0 μM of thionine as represented by curves (1–10). The expressed molar ellipticity (θ) values are based on polynucleotide concentrations. Representative induced circular dichroic spectra (lower panels) of 25 μM of thionine treated with 25, 50, 75, 100, 125, 150, 175, 200, 225, 250 μM of (E) poly(dA)·poly(dT) (F) poly(dA-dT)·poly(dA-dT) (G) poly(dG)·poly(dC) and (H) poly(dG-dC)·poly(dG-dC) as represented by curves (1–10). The expressed molar ellipticity (θ) values are based on thionine concentrations.

and is accessible to the quencher to the least extent with poly(dG-dC)·poly(dG-dC) and poly(dG)·poly(dC) where the binding appears to be true intercalation compared to the AT polynucleotides.

The specificity of the intercalative binding of thionine to the polynucleotides was also verified from viscosity measurements. A plot of the relative length enhancement versus r is presented in Fig. 4B. Viscosity results are expressed as length enhancement per base pair with respect to a standard value (β) of 1 corresponding to a length enhancement of 0.34 nm. The β values for thionine binding to poly(dG-dC)·poly(dG-dC), poly(dG)·poly(dC) poly(dA-dT)·poly(dA-dT) and poly(dA)·poly(dT) were 0.94, 0.83, 0.65 and 0.61 nm, respectively. Thus, a true intercalation scenario may be envisaged for the binding to the alternating GC base pair sequences of poly(dG-dC)·poly(dG-dC). This was followed by the homo GC sequences while a weaker intercalation process may be assigned for the binding to the AT polynucleotides in agreement with the fluorescence quenching results. Thus, viscosity experiments also clearly support the GC specificity of thionine.

3.4. Spectroscopic study using circular dichroism

The circular dichroic spectral pattern of the polynucleotides displayed a B-form conformation characterized by a large positive band in the 270–280 nm and a negative band around 245 nm although there are differences in the ellipticity and wavelength maxima. Conformational changes associated with the binding of thionine to these polynucleotides were investigated from circular dichroic studies. The CD bands of these polynucleotides are caused due to the stacking interactions between the bases and the helical structure that provide asymmetric environment for the bases. Thionine is an achiral molecule and does not have any optical activity but may acquire optical activity (induced CD) on binding to the helical organization of these polynucleotides. To record thionine-induced conformational changes, CD spectra in the 210–400 nm regions were recorded in presence of varying D/P (thionine/polynucleotide base pair molar ratio) values. In presence of thionine, the ellipticity of the long wavelength positive band of all the polynucleotides increased as the interaction progressed. The changes in the 275 nm band poly(dA)·poly(dT) were small but the positive 220 nm band and the negative 248 nm band ellipticities enhanced significantly. On the other hand, in the alternating AT polynucleotide only change was in the 275 nm band that red shifted and concomitantly enhanced in ellipticity. A new band due to the induced CD of thionine appeared around 310 nm that had a negative ellipticity. In the homopolymer of GC, there was a red shift of the long wavelength band and enhancement in the ellipticity. The 245 nm negative band also was perturbed significantly. In poly(dG-dC)·poly(dG-dC) there were enhancements in the ellipticity of both 275 and the 250 nm bands, but the change was more pronounced in the long wavelength band. The intrinsic CD spectral data are presented in Fig. 5A–D. It is apparent that the changes due to binding were not uniform and were significantly different. So the extent of structural change induced in the polynucleotides on binding appears to be different with different sequences but were within the B-conformation. Overall the changes were more pronounced in the GC polymers compared to the AT polynucleotides.

To examine the structural damage in more details, the induced CD spectra in the 300–700 nm region were investigated where neither the polynucleotides nor thionine have any CD spectra. This region exclusively presents the CD induced in the bound thionine molecules, if any, essentially on binding to the chiral DNA helix. Thionine molecules strongly bound may acquire strong induced circular dichroic characteristics in the asymmetric DNA environment. The induced CD spectral changes are presented in Fig. 5E–H. In poly(dA)·poly(dT) there was the formation of very weak induced CD bands. On the other hand, in the alternating AT polymer a large induced CD with a single peak was formed with maximum around 630 nm that enhanced as the binding progressed. The induced CD pattern with poly(dG)·poly(dC) had two peaks, a positive band around 630 nm band and a negative band around 575 nm that enhanced as the binding progressed. These bands are generated around the absorption band of thionine at 598 nm and appear to be of exciton split type. Similarly, with the alternating GC polymer there were only two bands of the excitation split type with maxima around 575 nm (negative) and 630 nm (positive) but the intensity was higher compared to that with the homo GC sequences. From these data it can be safely inferred that the orientation of the intercalated molecules in the various sequences of base pairs are different. It is known that planar dyes in the intercalated position can have an orientation with the long axis oriented either parallel (positive induced CD) or perpendicular (negative induced CD) to the long axis of the DNA. In poly(dA-dT)·poly(dA-dT) the sin-

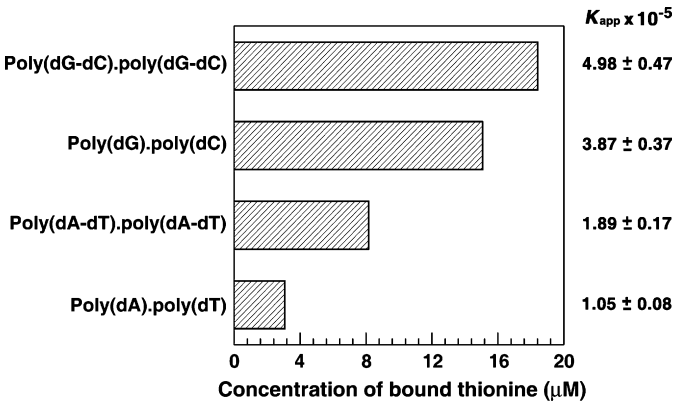

Fig. 6. The result of competition dialysis assay. The concentration of thionine bound to each polynucleotide sample is shown as a bar graph. The data given are average of four independent experiments under identical conditions.

gle positive induced CD compared to the exciton split bands in the GC polynucleotides suggests that the orientation of the intercalated thionines are clearly parallel to the long axis of the DNA. More detailed mechanism suggests that an effective electronic coupling between the bound dyes arranged to neighbouring sites that can induce a conservative pair of extrinsic CD on either side of the absorption band of the dye. The CD bands seen in the GC polymer may be attributed to an exciton splitting mechanism arising due to the effective interaction of the transition moments of intercalated thionine with that of the base pairs whereby the isohelical arrangement on the DNA results in an asymmetric arrangement of the dye chromophores. Although the induced CD patterns suggest different orientations in the AT polynucleotides compared to the GC polynucleotides for the intercalated thionine, conformation of the polynucleotide, sequence of base pairs, viz. AT versus GC sequences and factors like orientation, depth of intercalation, electrostatic contribution etc. also may contribute and control the fine features of the visible induced CD spectra. More detailed analysis is required to interpret the induced CD spectral patterns.

3.5. Competition dialysis assay

In order to unequivocally ascertain the overall sequence specificity of binding of thionine, the results of the competition dialysis assay of the four polynucleotide samples dialyzed against a common 1 μM thionine solution is presented as a bar graph in Fig. 6 where the concentration of thionine bound to each of the polynucleotide sample is plotted. Competition dialysis is an effective tool based on the fundamental thermodynamic principle of equilibrium dialysis developed by Chaires and coworkers [34,35] for the discovery of ligands that can bind with sequence specificity to DNA. The highest binding in terms of more accumulation of thionine was found to be with poly(dG-dC)·poly(dG-dC) (18.41 ± 1.31 μM) followed by poly(dG)·poly(dC) (15.08 ± 1.12 μM) and poly(dA-dT)·poly(dA-dT) (8.16 ± 0.65 μM). Poly(dA)·poly(dT) had the least preference as revealed by the lowest amount of bound thionine (3.09 ± 0.21 μM). The definitive conclusion that emerged from this experiment was the pronounced binding of thionine to the alternating GC polynucleotide followed closely by the homo GC polynucleotide sequences representing the next most preferred sequence. The binding preference varied in the order poly(dG-dC)·poly(dG-dC) > poly(dG)·poly(dC) ≫ poly(dA-dT)·poly(dA-dT) > poly(dA)·poly(dT). Thus, the AT sequences have significantly lower preference compared to the GC sequences and the homo AT sequences has the lowest binding preference. From the concentrations of bound dye, the apparent binding affinities (K_{app}) of thionine to the four polynucleotides were calculated. These values are 4.98×10^5, 3.87×10^5, 1.89×10^5 and $1.05 \times 10^5\ M^{-1}$, respectively, for the binding to poly(dG-dC)·poly(dG-dC), poly(dG)·poly(dC), poly(dA-dT)·poly(dA-dT) and poly(dA)·poly(dT). This result again confirms that the binding affinity of thionine was highest to the alternating GC sequences and the affinity varied in the order, GC/GC > G/C ≫ AT/AT > A/T sequences in complete agreement with that from the absorbance, fluorescence, circular dichroic and viscosity data.

4. Conclusions

This study presents the sequence specificity of the intercalative DNA damage of the phenothiazinium dye thionine with four sequence specific polynucleotides using various biophysical tools. The results revealed that thionine binds strongly with all the four polynucleotides; the strength of the binding being remarkably higher with the GC sequences compared to the AT sequences. The alternating sequences are preferred over the homo sequences. The binding was non-cooperative and the affinity of binding was highest with the GC alternating polynucleotide as determined from spectroscopic and competition dialysis assay. The binding resulted in significant perturbation of the conformation of the polynucleotides manifested by increase in the ellipticity of the positive and negative bands. The binding also resulted in the induction of strong optical activity for thionine molecules. The ellipticity of the induced CD bands was stronger with GC polynucleotides. Fluorescence quenching and hydrodynamic studies revealed that the binding of thionine to the polynucleotides was predominantly intercalative, again stronger with the alternating GC and homo GC polynucleotides compared to the alternating AT and homo AT sequences. Taken together, the results provide unequivocal evidences for the GC specificity of the intercalative DNA damage of thionine.

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