Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 189 (2018) 86–92

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Linear and circular dichroism characterization of thionine binding mode with DNA polynucleotides

Eimer Mary Tuite [a,*], Bengt Nordén [b]

[a] School of Chemistry, Bedson Building, Newcastle University, Newcastle upon Tyne NE1 7RU, UK
[b] Department of Chemical and Biological Engineering, Chalmers University of Technology, Gothenburg S-41296, Sweden

ARTICLE INFO

Article history:
Received 8 June 2017
Received in revised form 25 July 2017
Accepted 30 July 2017
Available online 1 August 2017

Keywords:
DNA binding
Phenothiazine dyes
Intercalation
Minor groove binding

ABSTRACT

The binding mode of thionine (3,7-diamino-5-phenothiazinium) with alternating and non-alternating DNA polynucleotides at low binding ratios was conclusively determined using linear and circular dichroism spectroscopies. The binding to $[poly(dG\text{-}dC)]_2$ and poly(dG)·poly(dC) was purely intercalative and was insensitive to ionic strength. Intercalative binding to $[poly(dA\text{-}dT)]_2$ is observed at low ionic strength, but a shift of some dye to an non-intercalative mode is observed as the background salt concentration increases. With poly(dA)·poly(dT), intercalative binding is unfavourable, although some dye molecules may intercalate at low ionic strength, and groove binding is strongly promoted with increasing concentration of background salt. However, stacking with bases is observed with single-stranded poly(dA) and with triplex poly(dT)*poly(dA)·poly(dT) which suggests that the unusual structure of poly(dA)·poly(dT) precludes intercalation. Thionine behaves similarly to the related dye methylene blue, and small differences may be attributed either to the ability of thionine to form H-bonds that stabilize intercalation or to its improved stacking interactions in the basepair pocket on steric grounds.

1. Introduction

Phenothiazinium dyes (Fig. 1) are widely used in biology for histological cell staining due to their metachromic behaviour [1] and have also found utility as redox transducers in biosensors [2]. Due to their photochemical activity in biological systems [3–5], they have potential applications in photodynamic therapy and this feature makes them suitable agents for photodecontamination of pathogens, for example when treating blood for transfusions [6]. Their biological activity is related, at least in part, to their nucleic acid binding properties. The most well-studied dye of this family is methylene blue (MB) which is full methylated on the exocyclic amines. We previously published comprehensive linear and circular dichroism studies of MB interactions with DNA and polynucleotides [7–9], which demonstrated intercalation of MB with most duplex DNA sequences as well as triplex poly(dT)*poly(dA)·poly(dT) [10]. However, non-intercalative binding occurred with poly(dA)·poly(dT), and also with $[poly(dA\text{-}dT)]_2$ at high ionic strength. Calculations by Rohs et al. [11] and Nogueira et al. [12] have indicated that MB has a preference for a minor-groove location in alternating AT sequences, with an angle between the dye transition moment and the helix axis that can produce zero linear dichroism signal, and this provides a theoretical underpinning of our observations with $[poly(dA\text{-}dT)]_2$. With $[poly(dG\text{-}dC)]_2$, modelling agrees with spectroscopic results that intercalation is favoured [13,14].

In thionine, the exocyclic amines are fully demethylated which alters the physical properties of the dye and also provides H-bonding possibilities. Although less well-studied than MB, the interaction of Th with DNA has gained increased attention in recent years [15–22]. We previously reported binding constants of MB and Th with calf thymus (CT-) DNA and alternating polynucleotides that were obtained by spectral analysis [15]. Th binds more strongly to all sequences, and both MB and Th show a small preference for binding to $[poly(dG\text{-}dC)]_2$ over $[poly(dA\text{-}dT)]_2$. However, there were indications of multiple binding modes for MB and Th with both these polynucleotides. Paul and coworkers explored Th binding to CT-DNA [16] by spectroscopy and also analysed binding thermodynamics with alternating and non-alternating polynucleotides using calorimetry [17]. They also found a preference for binding to $[poly(dG\text{-}dC)]_2$ compared to $[poly(dA\text{-}dT)]_2$ with a somewhat smaller binding constant for poly(dG).poly(dC). Calorimetry with poly(dA).poly(dT) gave anomalous results which suggests that the binding to this polynucleotide is unusual.

A major driver for studies of Th/DNA interactions is that Th is more readily reduced than MB and undergoes extremely efficient photoinduced electron transfer (PET) reactions with the nucleobases [23–25]. ^{1}MB emission is quenched by guanine but ^{1}MB quenching by adenine is generally prohibited by unfavourable energetics [26,27], although it is observed with poly(dA).poly(dT) [10]. Since the Th singlet state is

* Corresponding author.
E-mail address: eimer.tuite@ncl.ac.uk (E.M. Tuite).

http://dx.doi.org/10.1016/j.saa.2017.07.064

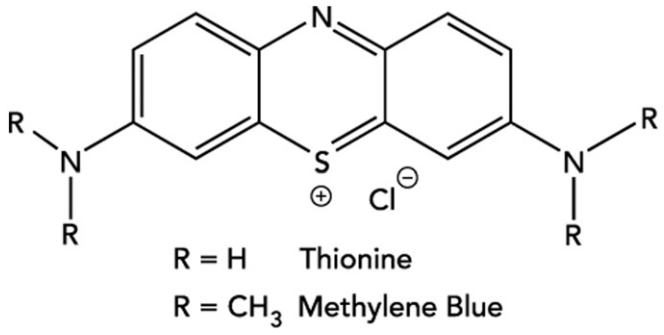

Fig. 1. Structures of the phenothiazinium dyes used in this study.

more oxidizing than MB, its emission is quenched by both guanine and adenine in all sequences [15]. The forward PET reaction between ^{1}Th and G is extremely efficient, resulting in a thousand-fold quenching of the excited state lifetime from 320 ps to 260 fs with no observable cage escape [23–25]. The ^{1}Th/A reaction is slower and produces a long-lived (>1 ns) transient [23,24], although it is not yet know how this is partitioned between the efficiently formed triplet state [28,29] and potential cage escape products.

Here, we report polarised spectroscopy studies for Th bound to several repetative DNA sequences, including triplex, duplex and single-stranded, to explore the influence of exocyclic amine methylation on the binding mode in order to facilitate fully interpretation of the photophysics.

2. Results

From UV/vis and emission spectra, it is known that thionine (Th) binds to alternating polynucleotides and CT-DNA with association constants on the order of 10^5–10^6 M^{-1} at low ionic strength [15–18]. Binding induces significant changes in absorption spectra with 10–15 nm red-shifts at the absorption maximum (600 nm) and 20–30% hypochromism. With $[poly(dG\text{-}dC)]_2$ at a fixed P/D, an isosbestic point was observed as ionic strength is increased, indicating a single binding mode. However, with $[poly(dA\text{-}dT)]_2$, absorption variations suggested a change of binding mode as ionic strength is raised [15]. In the present work, we explore the origins of these differences by characterising the binding modes of Th with alternating and non-alternating polynucleotides at low binding ratios (high P/D) using polarised spectroscopy at low and high ionic strength.

2.1. Binding to GC Polynucleotides

Fig. 2 shows the absorption, linear dichroism (LD), and induced circular dichroism (ICD) spectra for Th bound to $[poly(dG\text{-}dC)]_2$ under conditions where essentially all dye (>98%) is associated with polymer. The reduced linear dichroism (LD^r) signals are also shown as horizontal lines overlaying the spectra.

With $[poly(dG\text{-}dC)]_2$ at both high and low ionic strengths, the magnitude of the LD^r signal in the DNA absorption region (around 260 nm) drops when Th is bound, *i.e.* the orientation of the biopolymer decreases. The dye LD^r is more negative than the nucleobase LD^r, which is commonly observed for intercalation, possibly due to increased rigidity of the constrained helix at the binding site. CD spectra are shown in the visible region where only the dye absorbs. In general, interpretation of CD in the UV is complicated by convolution of free DNA, bound DNA, and bound dye spectra which precludes unambiguous interpretation of changes. In this case, at low binding ratios, none of the DNA spectra were perturbed on binding of Th (*e.g.* Fig. S3–S4) since the latter two factors are not significant. The ICD spectra at both ionic strengths are almost identical, and show the same bisignate features that were observed for MB bound to $[poly(dG\text{-}dC)]_2$ [8].

Spectra with poly(dG)·poly(dC) (supplementary material, Fig. S1) were similar to those with the alternating polynucleotide, with negative LD in the dye absorption band producing an LD^r signal for the dye that was equally negative as the bases, consistent with intercalation and concurring with the hole burning spectroscopy results of Hecht et al. [19,20]. The fluorescence intensity is efficiently quenched to a similar extent by both GC polynucleotides (0.11 with poly(dG)·poly(dC) *vs* 0.08 with $[poly(dG\text{-}dC)]_2$ at P/D = 46). Despite the different structures of the alternating and non-alternating polynucleotides [30], the spectral similarities indicate that the binding modes are both intercalative. Furthermore, the spectral variations observed for Th with these polynucleotides are almost identical to those previously reported for MB, showing that the exocyclic amines have little or no influence (either steric or H-bonding) on the interaction of phenothiazine dyes with GC-rich polynucleotides.

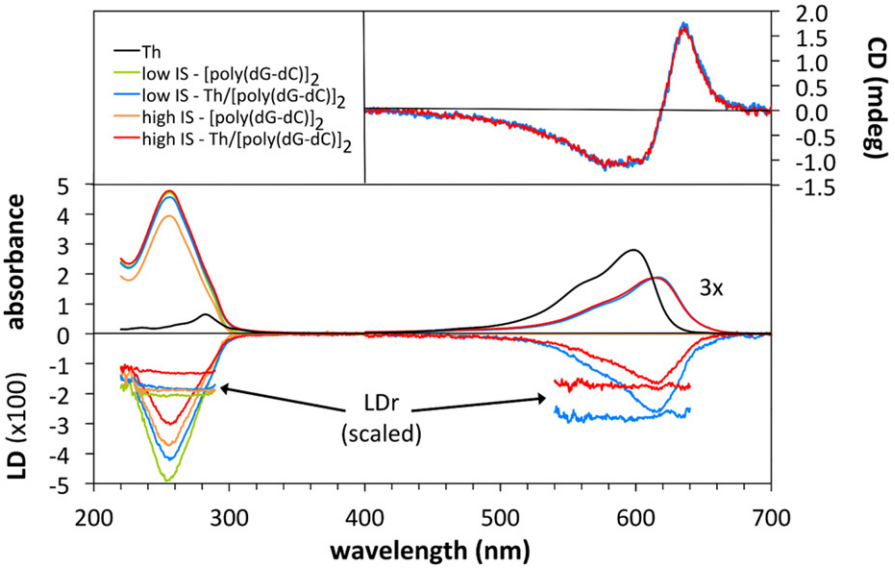

Fig. 2. Absorption, LD, LD^r, and ICD spectra for Th with $[poly(dG\text{-}dC)]_2$. [Th] = 13 μM. P/D = 46. Low ionic strength = 5 mM phosphate (pH 6.9); high ionic strength = 200 mM phosphate (pH 6.9).

2.2. Binding to AT Polynucleotides

Fig. 3 shows the absorption, linear dichroism (LD), and induced circular dichroism (ICD) spectra for Th bound to $[poly(dA\text{-}dT)]_2$ at low binding ratio (high P/D).

With $[poly(dA\text{-}dT)]_2$ at both ionic strengths, the LD^r of the dye is more negative than that of the nucleobases, consistent with intercalation. For both samples, the orientation of the polynucleotide increases when the dye binds, *i.e.* the LD^r signal at about 260 nm increases in magnitude. ICD spectra are bi-signate and the spectrum at low ionic strength is very similar to that observed with $[poly(dG\text{-}dC)]_2$. However, with this polynucleotide, the ICD spectrum varies significantly with background salt and the negative lobe drops in intensity as the ionic strength is raised. This is consistent with a shift of binding mode, as previously indicated by the lack of an isosbestic point in absorption spectra as the ionic strength is raised [15]. We note that the ICD spectra for Th with $[poly(dA\text{-}dT)]_2$ have a different band shape than those observed for MB with $[poly(dA\text{-}dT)]_2$ which are entirely positive. This suggests that the orientation of the dye in the binding pocket is sensitive to exocyclic amine substitution [15].

With poly(dA)·poly(dT) (Fig. 4), the absorption and LD^r spectra are more sensitive to ionic strength than for any other polynucleotide, although the changes are not as dramatic as those observed with MB [9]. As background salt increases, absorption shows a shift to the blue with greater hypochromism and no isosbestic point in absorption, which indicates a change of binding mode as well as increased free dye concentration. The negative dye LD^r signal is smaller in magnitude than the nucleobase signal and it drops as the ionic strength is raised although it does not disappear. With other polynucleotides, the absorption and LD spectra have the same band-shape and maxima. However, with poly(dA)·poly(dT), the LD maxima are red-shifted compared to the absorption bands, which gives rise to a wavelength-dependent LD^r which is particularly marked at high ionic strength where the absorption blue-shifts although the LD does not. This suggests that multiple bound species with disparate spectral characteristics co-exist, and that they are differently responsive to changing ionic strength. Despite being negative, the LD^r signals are not characteristic of intercalation, particularly at high ionic strength, so non-intercalative binding modes must occur. To distinguish whether the non-intercalative mode is groove-binding or electrostatic binding to the backbone, we must compare LD results with ICD. The ICD spectra with poly(dA)·poly(dT) are entirely different from those observed with $[poly(dA\text{-}dT)]_2$. They have large magnitude and are positive across the absorption band, consistent with groove-binding [31], similar to the ICD spectra observed for MB with this polynucleotide [9]. The ICD maximum blue-shifts with increasing ionic strength in line with absorption shifts.

With mixed sequence CT-DNA, the spectra show elements of the spectra observed with the different alternating and non-alternating polynucleotides (supplementary material, Fig. S2). Like $[poly(dG\text{-}dC)]_2$, the DNA orientation decreases on dye binding and the more negative LD in the dye band is consistent with intercalation, while the ICD spectra resemble the bisignate spectra observed with both alternating polynucleotides. Like $[poly(dA\text{-}dT)]_2$, the spectra change with increasing ionic strength.

In the presence of excess poly(dT) and Mg^{2+}, poly(dA)·poly(dT) becomes a non-alternating triple helix [poly(dT)*poly(dA)·poly(dT)], where poly(dT) forms Hoogsteen bonds with the adenine bases in the major groove [10,32]. Under our experimental conditions (20 mM phosphate/2 mM Mg^{2+}), this triplex is intact at 20 °C (T_m = 33.4 °C). The stability of these helices in the presence of Th was investigated by monitoring the CD of DNA in the UV region (Figs. S3–S8). The triplex is strongly stabilised by addition of Th ($\Delta T_m = +12.2$), even though the dye has little effect on the stability of the precursor duplex ($\Delta T_m = +1.3$), as shown in Table 1 (see also the Figures in the supplementary material). For the Th/triplex complex, LD^r of the dye is more negative than the DNA which is consistent with Th intercalation, as previously observed for MB [10]. The ICD spectrum of Th with poly(dT)*poly(dA)·poly(dT) is negative, indicating different coupling between the base triplets and the dye than occurs in the parent duplex where binding is likely non-intercalative.

Th also binds to single-stranded poly(dA) at low ionic strength (5 mM phosphate), as shown by hypochromism and a red-shift in absorption (Fig. 5). Poly(dA) has a large negative LD signal at low ionic strength, showing that the chain is well oriented and that the bases lie on average at between 55° and 90° to the helix axis. Binding of Th increases the polynucleotide alignment, and bound Th has a negative LD spectrum with dye LD^r being more negative than the base LD^r, consistent with stacking between adjacent bases of the single strand in a pseudo-intercalative mode (note that there is no indication in the DNA CD spectra that Th induces duplex formation). A negative ICD spectrum is observed, which resembles the spectrum with triplex poly(dT)*poly(dA)·poly(dT). At moderate ionic strength (100 mM

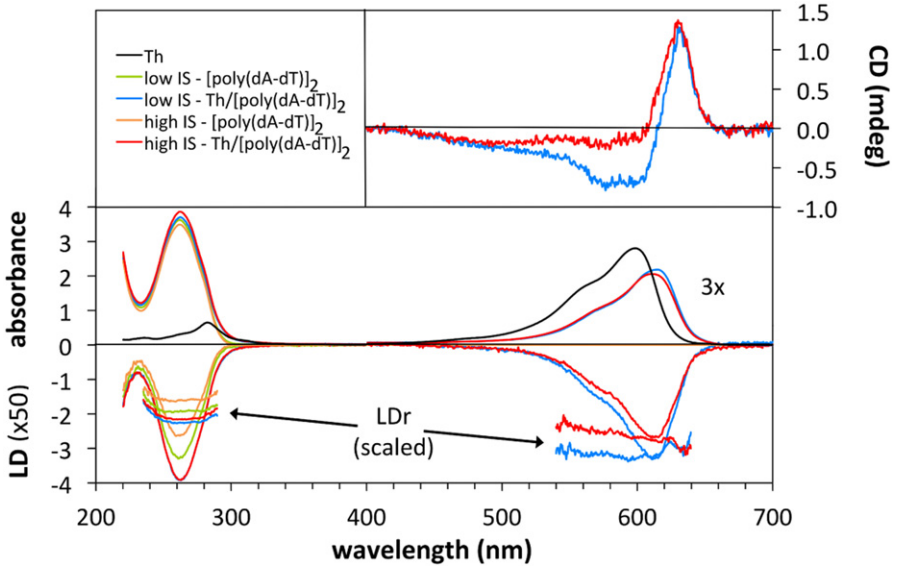

Fig. 3. Absorption, LD, LD^r, and ICD spectra for Th with $[poly(dA\text{-}dT)]_2$. [Th] = 13 μM. P/D = 46. Low ionic strength = 5 mM phosphate (pH 6.9); high ionic strength = 200 mM phosphate (pH 6.9).

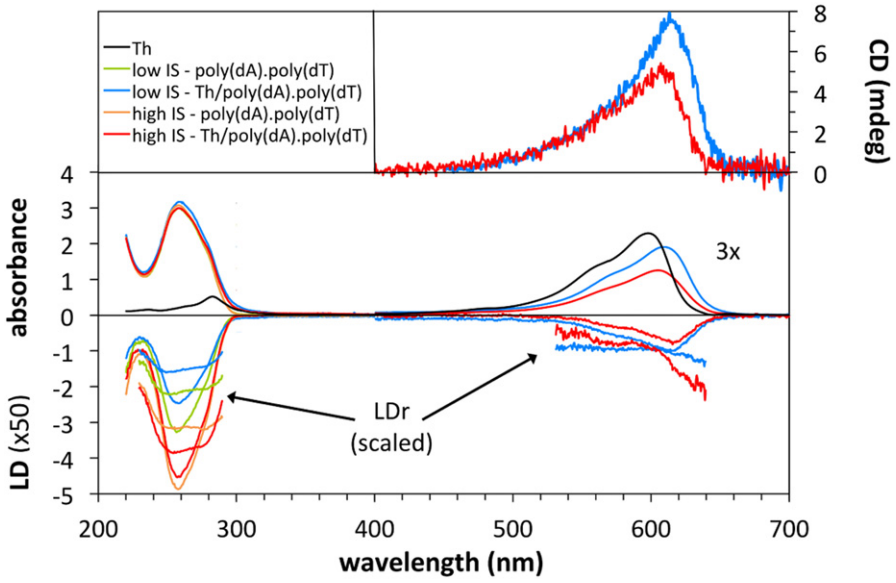

Fig. 4. Absorption, LD, LD^r, and ICD spectra for Th with poly(dA).poly(dT). [Th] = 10 μM. P/D = 60. Low ionic strength = 5 mM phosphate (pH 6.9); high ionic strength = 5 mM phosphate (pH 6.9)/100 mM NaCl.

NaCl), binding to poly(dA) is completely eliminated and no fluorescence quenching is observed, demonstrating the importance of columbic forces for stabilization of the binding.

3. Discussion

De-methylation of the MB exocyclic nitrogen's to give Th leads to substantial changes in the chemistry of the phenothiazine dye, making it more oxidizing and making its singlet state more energetic. Thus, photoinduced electron transfer to excited state thionine from DNA purine bases is very favorable. This study aimed to explore whether demethylation also influences the binding of Th to different DNA sequences. Such information is essential for correct interpretation of photo physical and photochemical studies of Th bound to DNA. Previous work with UV/vis spectroscopy [15] indicated that Th binds more strongly than MB to DNA, but found little selectivity between sequences. However, UV/vis spectroscopy cannot determine either the location of the dye, *i.e.* intercalated or groove-bound. The combination of linear and circular dichroism used in the current study elucidates how bind can depend on sequence, dye structure, and environmental conditions. We find that the binding of Th to alternating and non-alternating polynucleotides, and to mixed natural sequence CT-DNA, is similar to that previously reported for MB, although there are notable differences in the behaviour with exclusively AT sequences.

LD^r results demonstrate that binding to [poly(dG-dC)]$_2$ and poly(dG)·poly(dC) are clearly intercalative, irrespective of ionic strength. A bisignate ICD for Th was observed with [poly(dG-dC)]$_2$, the origin of which has been discussed previously for MB [7–9]. Tjerneld [7] suggested that this arose from two orthogonal intercalation orientations, and Lyng et al. [8] described how the sign of the ICD indeed depends sensitively on both the lateral and rotational displacement within the intercalation pocket as well as on the nature of the surrounding basepairs. Alternatively, it was suggested for MB that bisignate ICD spectra could alternatively arise from partitioning of intercalated dye between the two base pair steps [9], since the ICD with poly(dG)·poly(dC) is entirely negative. However, for Th we see that ICD spectra with [poly(dG-dC)]$_2$ and poly(dG)·poly(dC) are very similar and both bisignate, so the original proposal of Tjerneld that there is more than one intercalation geometry is the most appropriate model.

With [poly(dA-dT)]$_2$, the dye LD^r is more negative than the DNA LD^r at both high and low ionic strength, which is consistent with intercalation. However, the LD^r(dye)/LD^r(DNA) changes from 1.43 to 1.28 as the ionic strength increases. Since the absorption variation associated with increasing ionic strength does not show an isosbestic point, this implies a change of binding mode for a small proportion of Th with increasing ionic strength. This is a modest effect compared to MB at high ionic strength where a large positive ICD and near zero LD (*i.e.* with the dye plane orientated just below the magic angle wrt the helix axis) imply that essentially all bound dye occupies a minor groove site, shown also by modelling [11,12]. The bi-signate ICD spectra for Th are very different from the positive spectra for MB, but a small change in bisignate shape with increasing ionic strength may reflect some Th shifting from intercalation to groove binding. It is unclear why MB and Th have different spectroscopic signatures with [poly(dA-dT)]$_2$ while they give almost identical spectra with [poly(dG-dC)]$_2$. Although there are several molecular modelling studies of MB with DNA and alternating polynucleotides [11–14], there are no similar works regarding Th which could help to explain the spectral variations in more detail.

With poly(dA)·poly(dT), Th spectra are again similar to those observed with MB. At both ionic strengths, the DNA LD^r is more negative than the Th LD^r although, in both conditions, the dye LD^r has a larger magnitude than reported previously for MB. Nonetheless, the LD^r(dye)/LD^r(DNA) drops substantially from 0.6 to 0.2 as the ionic strength increases while absorbance decreases and ICD remains large and positive (although shifted). These results are not consistent with classical intercalation and, as discussed for MB [9], binding may be a mixture of intercalation and groove binding. Alternatively, Th may be groove-bound under all conditions with an ionic strength-dependent orientation that gives rise to a negative LD. Certainly, the ICD intensities are much larger than those observed with the alternating

Table 1
The effect of Th on the thermal denaturation of duplex poly(dA)·poly(dT) and triplex poly(dT)·poly(dA)·poly(dT) measured by circular dichroism.

	T_m (ds → ss)/°C Duplex	T_m (ds → ss)/°C Triplex	T_m (ts → ds)/°C Triplex
No dye	64.9	65.4	33.4
Th	66.2	66.7	45.7
ΔT_m/°C	*+1.4*	*+1.3*	*+12.3*

[Th] = 6 μM; [poly(dA).poly(dT)] = 100 μM base; [poly(dT)·poly(dA)·poly(dT)] = 150 μM base; buffer = 20 mM phos/2 mM $MgCl_2$.

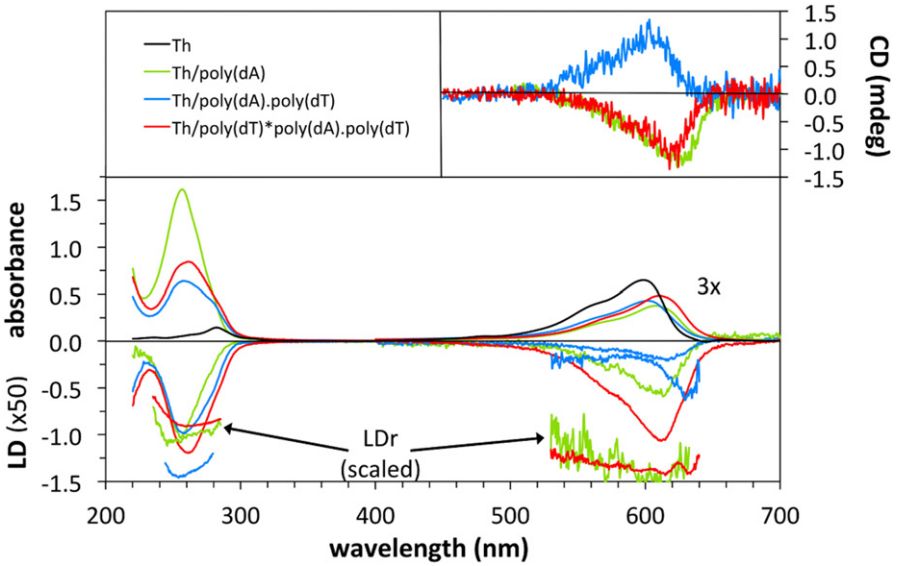

Fig. 5. Absorption, LD^r, and ICD spectra for Th with poly(dT)·poly(dA)·poly(dT) and poly(dA) compared to poly(dA)·poly(dT). [Th] = 3 μM. [poly(dA)] = 180 μM base; buffer = 5 mM phosphate (pH 6.9). [poly(dA).poly(dT)] = 127 μM base; [poly(dT)·poly(dA)·poly(dT)] = 143 μM base; buffer = 20 mM phosphate (pH 6.9)/2 mM Mg^{2+}.

polynucleotides, and are of a magnitude that is consistent with groove binding [8,31]. For MB, it was impossible to discern whether intercalation was blocked by steric effects from the methyl substituents on the exocyclic amines or by the inability of the polynucleotide to accommodate intercalation. Since Th lacks the methyl groups, this indicates that it is the unusual structure of poly(dA)·poly(dT) [29] that provides an insurmountable hurdle to intercalation of phenothiazine dyes. Crescent-shaped, flexible minor groove binders such as distamycin and netropsin bind strongly in A.T-tracts, where the groove is narrow and provides a deep binding pocket. However, certain linear molecules have been observed to bind in the minor groove of A.T-tracts, facilitiated by interfacial water molecules [33]. Th and MB can be envisaged to bind to the minor groove of poly(dA)·poly(dT) in this manner. The larger values of LD^r(dye)/LD^r(DNA) for Th with poly(dA)·poly(dT) compared to MB may be explained by the H-bonding functionality of the Th amines that could produce different a different bound orientation in the groove with a different LD response.

Notably, when a poly(dT) third strand is added to the major groove of poly(dA)·poly(dT) to form a triplex structure, LD spectra consistent with Th intercalation are observed, and the ICD changes sign to become negative. Very similar spectra are observed when Th interacts with poly(dA) at low ionic strength which we interpret as Th stacking between adjacent A bases in the single strand. All these results converge to show that the poly(dA)·poly(dT) structure is not conducive to intercalation, despite it being found to adopt a B-like structure [30]. It is instructive at this point to compare our findings on the binding of Th to DNA non-alternating AT sequences with those previously reported for analogous RNA sequences; the behaviours are diametrically opposite. Paul et al. [34] observed a negative ICD for Th with poly(A) which they concluded was due to intercalation between base-pairs in a self-assembled poly(A) duplex. This was supported by spectroscopic and thermal results that showed that the duplex and its complex with Th persisted at higher ionic strengths, contrary to the absence of Th/poly(dA) binding that we observe at high salt. The differences between binding to DNA and RNA are further apparent by considering the report of Lozano et al. [35] in which Th destabilises triplex poly(U)·poly(A)·poly(U) where it lies in the groove but stabilises duplex poly(A)·poly(U) where it is intercalated, resulting in an isothermal denaturation of triplex to duplex and single strand which is the opposite behaviour to our comparable DNA triplex *versus* duplex results. Thus, even a simple molecule like Th, like its phenazine analogue proflavine [36,37], can exhibit a range of binding modes with nucleic acids and can show strong selectivity in binding to different nucleic acid structures, sequences, and conformations.

4. Conclusions

In conclusion, this study finds that removing the exocyclic *N*-methyl groups from methylene blue (MB) to produce thionine (Th) has moderate effects on the binding of the dyes to DNA polynucleotides. Binding of Th to alternating or non-alternating GC polynucleotides is fully intercalative and the binding mode is insensitive to ionic strength, similar to MB. Binding to AT-only polymers was influenced by methylation of the external amines, particularly regarding the effects on ionic strength. With [poly(dA-dT)]$_2$, the influence of increasing ionic strength in promoting a shift from intercalative to non-intercalative binding was less for Th than for MB. LD with poly(dA).poly(dT) suggested that some Th intercalation might occur at low ionic strength, but that non-intercalative binding – likely in the minor-groove – was strongly promoted as ionic strength increased, although less dramatically than for MB. It is possible that the stronger binding of Th compared to MB might arise from H-bonding to phosphate groups that could stabilize the intercalative mode, or that steric hindrance between the methyl groups of MB and the base pair edges might prevent ideal stacking in the intercalative pocket. Since stacking between A and T bases is favorable in single-stranded poly(dA) and in triplex poly(dT)·poly(dA)·poly(dT), the non-intercalative binding mode with poly(dA)·poly(dT) likely arises from an unusual structure of this polynucleotide that precludes easy insertion of the dyes between the base-pairs.

5. Experimental

5.1. Materials

Buffers were prepared using analytical grade dry Na_2HPO_4, NaH_2PO_4, NaCl and $MgCl_2$ (Fluka); phosphate buffers of pH 6.9 were prepared at different concentrations by mixing equal molar quantities of Na_2HPO_4 and NaH_2PO_4. Water was purified with a Millipore Milli-Q system. Experimental samples were prepared using calibrated micropipettes. Methylene blue (Fluka puriss, 98%) and thionine (Aldrich, 91%) were purified by chromatography on Sephadex LH-20 with methanol as eluent. Multiple separations were required, and purity was confirmed by tlc on silica with 9:1 methanol/acetic acid. Equivalence of absorption

and excitation spectra confirmed the absence of significantly emissive impurities. Dye concentrations were determined using the following molar extinction coefficients in water: MB, $\varepsilon_{664} = 81{,}600\ M^{-1}\ cm^{-1}$; Th, $\varepsilon_{598} = 71{,}500\ M^{-1}\ cm^{-1}$ [38].

High molecular weight calf thymus DNA (Type I, sodium salt, Sigma) was dissolved in pure water and dialysed extensively against 5 mM phosphate buffer (pH 6.9). The synthetic polynucleotides [poly(dG-dC)]$_2$, [poly(dA-dT)]$_2$, poly(dG)·poly(dC), poly(dA)·poly(dT), and poly(dA) were purchased from Pharmacia and supplied as lyophilised solids. [poly(dG-dC)]$_2$, [poly(dA-dT)]$_2$, and poly(dA) were reconstituted in 5 mM phosphate (pH 6.9). Poly(dG)·poly(dC) and poly(dA)·poly(dT) were reconstituted in 100 mM NaCl/5 mM phosphate, as recommended by the manufacturer. Alternatively, duplex sequences were synthesised in-house using published methods [39]. Concentrations were determined using the following molar extinction coefficients in phosphate buffer: CT-DNA, $\varepsilon_{260} = 6600\ M^{-1}\ cm^{-1}$; [poly(dG-dC)]$_2$, $\varepsilon_{254} = 8400\ M^{-1}\ cm^{-1}$; [poly(dA-dT)]$_2$, $\varepsilon_{262} = 6600\ M^{-1}\ cm^{-1}$; poly(dG)·poly(dC), $\varepsilon_{253} = 7400\ M^{-1}\ cm^{-1}$; poly(dA)·poly(dT), $\varepsilon_{260} = 6000\ M^{-1}\ cm^{-1}$; poly(dA), $\varepsilon_{257} = 8600\ M^{-1}\ cm^{-1}$. All nucleic acid concentrations are presented in terms of nucleotide concentration. Triplex poly(dT)·poly(dA)·poly(dT) was prepared by mixing a 2:1 molar ratio of poly(dT) and poly(dA) in 20 mM phosphate (pH 6.9)/2 mM $MgCl_2$, incubating with stirring at 80 °C for 30 min, and slowly cooling the solution to room temperature [40].

5.2. Spectra

All spectra were normalized to a 1 cm pathlength, and were measured at ambient temperature which was controlled to be 18–21 °C. Isotropic UV/vis absorption spectra were measured with a Varian Cary 2300 spectrometer. Circular dichroism (CD) spectra (the differential absorption of left and right circularly polarised light) were measured on a JASCO J-720 instrument. Although the dyes are achiral, they exhibit induced CD (ICD) signals when bound to the helical nucleic acids due to coupling of their electric dipole transition moments to the transition moments of the chirally organised nucleobases. The data are presented as they were collected in mdeg; these can be converted to absorbance units through division by a factor of 32,980 mdeg [41].

$$CD\ (\lambda) = A_{left}\ (\lambda) - A_{right}\ (\lambda)$$

Thermal denaturation of triplex DNA was measured using CD with a peltier heater.

Linear dichroism (LD) spectra were measured on a JASCO J-500A spectropolarimeter, adapted with an Oxley prism to convert incident circularly polarised light to linearly polarised light. Sample orientation was achieved in a flow Couette cell with an outer rotating cylinder and an experimental pathlength of 1 mm. LD is the differential absorption of light polarised parallel and perpendicular to the flow direction (the helical axis of DNA) in a Couette cell. The magnitude of the LD signal depends on the degree of orientation of the sample, as well as the molar absorptivity and concentration of the sample.

$$LD\ (\lambda) = A_{parallel}\ (\lambda) - A_{perpendicular}\ (\lambda)$$

The reduced linear dichroism (LD^r) [41] is the ratio of LD and isotropic absorption signals, and is related to the orientation of the chromophore:

$$LD^r\ (\lambda) = LD\ (\lambda)/A_{iso}\ (\lambda) = 3/2\ \{S\ (3\langle\cos^2\alpha\rangle - 1)\}$$

where α represents the angle between the absorbing transition moment and the DNA helical axis. S is an orientation function that describes the extent of DNA orientation such that $S = 1$ represents perfect orientation and $S = 0$ represents random orientation. S depends on DNA stiffness, the flow rate, and the viscosity of the medium. S can be determined from the dichroism of DNA at 260 nm where the π-π* transitions are polarised in the plane of the nucleobases. Previous linear dichroism results indicate an effective angle of 86° between the average nucleobase plane and the helix axis for B-DNA [42] or 72° using electric linear dichroism [43].

Abbreviations

CD	circular dichroism
ICD	induced circular dichroism
LD	linear dichroism
LD^r	reduced linear dichroism
MB	methylene blue
Th	thionine

Acknowledgements

This work was supported by the Swedish Research Council (VR) (NFR/212221) and the Engineering and Physical Sciences Research Council UK (EPSRC GR/S23315/01). Data supporting this publication is openly available under an 'Open Data Commons Open Database License'. Additional metadata are available at: http://dx.doi.org/10.17634/021706-2. Please contact Newcastle Research Data Service at rdm@ncl.ac.uk for access instructions.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.saa.2017.07.064.

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