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Structural Heterogeneity in Polynucleotide-Facilitated Assembly of Phenothiazine Dyes

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ABSTRACT: The assembly of stacked dyes on DNA is of interest for electron transfer, light harvesting, sensing and catalysis applications. This paper addresses the unresolved role of stacked phenothiazine dyes in photosensitization by determining the structures of aggregates on DNA as a function of dye substituents and polynucleotide sequence. A combination of UV/vis absorption, linear dichroism (LD), and circular dichroism (CD) was applied to characterize thoroughly the aggregation with DNA of the phenothiazine dyes methylene blue, azure B, and thionine. Aggregates of each dye with [poly(dG-dC)]₂, [poly(dA-dT)]₂ and DNA were explored at low P/D ratio, where dyes bind in the grooves when all intercalation sites are saturated. The

organization of the aggregates (dimers, trimers and multimers) with polydeoxynucleotides displays a structural diversity that depends on DNA sequence, extent of methylation of dye exocyclic amine groups, and ionic strength. The dyes typically form right-handed H-aggregates having negative LD, consistent with stepped stacking along the minor groove. However, a few aggregates show left-handed chirality or positive LD, indicating unusual modes of aggregation such as formation of adventitious dimers between intercalated and minor groove bound dye molecules. In terms of sequence-dependence, methylene blue shows more extensive aggregation with [poly(dA-dT)]₂ while thionine aggregates more with [poly(dG-dC)]₂. Azure B has distinctive behavior that is unlike either other dye. Thus, although these phenothiazine dyes possess a common tricyclic framework, the organization of their polynucleotide-facilitated aggregates depends sensitively on the extent of methylation of the exocyclic amines.

INTRODUCTION

Phenothiazine dyes (Figure 1) are widely used in biology as histological stains due to their sensitive metachromic effects – this is due to varying degrees of aggregation when the dyes bind to different biopolymers in organelles. These dyes also have applicability in photodynamic therapy¹ and photosterilization of blood² owing to their high singlet quantum oxygen yields coupled with their ability induce oxidative damage in biomolecules such as DNA and proteins.³ The role of phenothiazine aggregates in photodynamic reactions remains unresolved. Assembly of phenothiazine photosensitizers has been variously reported to reduce photodynamic effects in mitochondria⁴ or to enhance photosensitized inactivation of bacteria.⁵ However, time-resolved spectroscopy of the methylene blue dimer in solution shows complete fast relaxation to the

ground state (~ 10 ps) which indicates that it cannot participate in slow photodynamic processes.^{6,7} To facilitate understanding of such in vivo processes, we use DNA as an in vitro model to study the interplay between photochemical damage and dye aggregation processes. In this study we characterize phenothiazine dye aggregation as a function of dye structure, DNA sequence, and ionic strength.

Figure 1. Structures of the studied phenothiazine dyes, showing the direction of the lowest energy long-axis polarized transition moment.

Cofacial self-association of planar aromatic dyes occurs in aqueous solution when strong intermolecular attraction forces coupled with the hydrophobic effect result in dye-dye interactions becoming more favorable than dye-solvent interactions. Charged dyes such as phenothiazines experience electrostatic repulsion that limits self-association when dilute, but dimerization is favored as the concentration of dye or the background ionic strength are raised.^{8,9} Higher order stacking is observed at high dye concentrations¹⁰⁻¹³ or when the dyes are adsorbed on surfaces,¹⁴⁻¹⁶ in microheterogeneous environments,^{17,18} or bound to polyanions.¹⁹⁻²² Molecular aggregates are usually identified quantitatively by changes in optical spectra since the coupling of transition dipole moments in a stacked system gives rise to different photophysical characteristics compared to the monomer, e.g. exciton coupling, altered band widths, energy shifts in UV/vis and fluorescence spectra, and modified emission quantum yields and lifetimes.

Exciton theory explains the hypsochromic (blue-) shift of H-aggregates (side-by-side orientation) and the bathochromic (red-) shift of J-aggregates (slipped or head-to-tail orientation), which were initially identified for pseudoisocyanine.²³ The photophysical, redox and energy transfer properties, and the biological activity of stacked dyes have applications in areas such as photodynamic therapy, DNA-targeting, optical sensors, dye-sensitized solar cells and optoelectronics. For example, the distinctive color changes associated with cyanine dye stacking on DNA and peptides have been exploited for development of genetic sensors.^{24,25} Moreover, assembly of small molecules (which are more readily delivered to *in vivo* targets) at specific targets²⁶ provides potential new recognition tools in diagnostic and therapeutic contexts, including DNA sequence recognition.

Methylene blue (MB), Azure B (AB), and thionine (Th) are the phenothiazine dyes studied in this work.^Δ The aggregation of MB in solution and at interfaces has been well-characterized by UV/vis spectroscopy. Monomeric MB exists in dilute (micromolar) aqueous solution and exhibits a long axis-polarized π - π^* transition at 664 nm with a vibronic (0-1) shoulder at 615 nm.⁸⁻¹⁴ MB dimers have an absorption maximum at 605 nm with a lower intensity peak around 690 nm,⁸⁻¹⁴ consistent with face-to-face stacking with variously a 13°¹⁴ or 32°²⁷ angle between transition moments (Figure 2). The trimer is characterized by a maximum near 570 nm^{10,11,15} and is observed at high concentrations (ca. 0.1 M) in solution. Higher aggregates are rarely observed in solution but recently Heger and coworkers reported absorption bands of aggregates bigger than trimer in frozen aqueous solutions where the local concentration of dye is

^Δ Azure C and Azure A were unstable during experiments at neutral pH in solution, particularly when bound to DNA, so were not included in this study. Both dyes decomposed to other phenothiazines and side-products, even in the dark.

dramatically increased compared to solution.¹² These spectra after slow freezing to 243 K show absorption of the higher order H-aggregate at about 510 nm with a shoulder at 470 nm and provide important information for characterization of aggregate size. The aggregation of Th^{8,11,13,15,27} and AB²⁷⁻²⁹ are less well studied but their aggregation behaviors are similar to MB.

Figure 2. Proposed structure of methylene blue dimer in aqueous solution. The angle between the long-axis polarized transition moments has been calculated to be variously 13°¹⁴ and 32°²⁷.

Th monomer in water absorbs at 597 nm with a vibronic shoulder at 560 nm.^{8,11,13} A peak at 557 nm has been assigned to dimer^{8,26} with a low intensity band at 630 nm.²⁷ Other studies assigned a band at about 560 nm not just to dimer and aggregates of several units.^{11,13} No distinct bands were observed for aggregates of larger size, but a continuous blue-shift was observed to 540 nm (9 units).¹¹ Calculations by Lai et al. suggest that an infinite aggregate should have an absorption maximum at 533 nm.¹¹ Bands have been observed at 545 nm with a mesoporous material¹⁵ and at 525 nm with a colloid¹⁶ which have been assigned to H-aggregates.

AB monomer has a maximum absorbance at 646 nm with a vibronic shoulder at 598 nm. Dimer absorption has been reported at 588 nm with a low intensity peak at 682 nm in aqueous solution.²⁷ Spectra of higher aggregates have not been reported in water but have been variously reported on binding to the polysaccharides chondroitin²⁸ (597 nm = dimer, 555 nm = higher order aggregates), and heparin (556 nm = aggregate) and alginate (545 nm = aggregate).²⁹

Phenothiazine dyes typically intercalate between DNA base pairs when the nucleic acid is in excess, but can also occupy groove-bound sites in certain sequences.³⁰⁻⁴⁵ They are known to stack externally on the polyanionic DNA backbone when DNA is in excess,^{19-22,30-32} but neither the organization of dyes in the aggregates nor the effects of sequence or dye structure has received significant attention. Methylation of the exocyclic amines has little effect on intercalative binding at high P/D.^{30,32} However, the hydrogen bonding potential of Th and AB, as well as their different steric properties, likely influences their external stacking interactions. This study explores this hypothesis and also demonstrates the role that DNA sequence plays in controlling the stacking orientations of these dyes.

EXPERIMENTAL

Materials. Buffers were prepared using analytical grade dry Na_2HPO_4 and NaH_2PO_4 (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%), thionine (Aldrich, 91%), and azure B (Aldrich, ~ 84%) 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, $\epsilon_{664} = 81,600 \text{ M}^{-1} \text{ cm}^{-1}$; Th, $\epsilon_{598} = 71,500 \text{ M}^{-1} \text{ cm}^{-1}$; AB, $\epsilon_{647} = 78,000 \text{ M}^{-1} \text{ cm}^{-1}$.³⁷

High molecular weight calf thymus DNA (Type I, sodium salt, Sigma) was dissolved in pure water and dialyzed extensively against 5 mM phosphate buffer (pH 6.9). The synthetic

polynucleotides [poly(dG-dC)]₂ and [poly(dA-dT)]₂ (GE Healthcare) were supplied as lyophilized solids and reconstituted in 5 mM phosphate (pH 6.9). Alternatively, duplex sequences were synthesized in-house using published methods.⁴⁶ Concentrations were determined using the following molar extinction coefficients in phosphate buffer: CT-DNA, $\epsilon_{260} = 6,600 \text{ M}^{-1} \text{ cm}^{-1}$; [poly(dG-dC)]₂, $\epsilon_{254} = 8,400 \text{ M}^{-1} \text{ cm}^{-1}$; [poly(dA-dT)]₂, $\epsilon_{262} = 6,600 \text{ M}^{-1} \text{ cm}^{-1}$. All nucleic acid concentrations are presented in terms of nucleotide concentration.

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 polarized light) were measured on a JASCO J-720 instrument. 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.⁴⁷

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

Linear dichroism (LD) spectra were measured on a JASCO J-500A spectropolarimeter, adapted with an Oxley prism to convert incident circularly polarized light to linearly polarized 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 polarized 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.

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

LD is related to the isotropic absorption and to the orientation of the chromophore:⁴⁷

$$\text{LD}(\lambda) = [A_{\text{iso}}(\lambda)] \frac{3}{2} \{S(3\langle\cos^2\alpha\rangle - 1)\}$$

where α represents the angle between the absorbing transition moment and the DNA helix 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 polarized in the plane of the nucleobases. Previous work indicates an effective angle of 86° between the average nucleobase plane and the helix axis for B-DNA using flow linear dichroism⁴⁸ or 72° using electric linear dichroism.⁴⁹

Methodology. In our LD experiments, the orientation axis is parallel to the DNA helix (Figure 3). The sign and magnitude of an LD signal reflects the angle of orientation and the extent of orientation of an anisotropic sample. DNA exhibits a negative LD spectrum in the UV region that mirrors its absorption, since the transition moments (TMs) in the nucleobase planes are oriented approximately perpendicular to the helix axis.^{48,49} Dye transition moments at 54.74° (magic angle) to the DNA helix axis have no LD signal, whilst those oriented at smaller angles have positive LD (e.g. those aligned along the minor groove at 45°), and those at larger angles have negative LD (e.g. those intercalated between base pairs). Dye LD signals arise solely from molecules that are aligned when they bind to DNA. Differences between absorption and LD can arise from unbound (isotropic) species manifesting in the absorption spectrum but not contributing to LD. Other differences between absorption and LD occur when distinct bound species have dissimilar orientation angles (α) or orientability (S).

Figure 3. Principles of linear dichroism for shear-oriented DNA, and circular dichroism for stacked dimers.

In CD, signals can arise from (i) intrinsically chiral species in solution e.g. helical dimers or aggregates of dyes, (ii) non-chiral species which experience induced CD (ICD) on binding to helical DNA e.g. intercalators and monomeric groove binders, (iii) intrinsically chiral species bound to DNA, and (iv) new chiral arrangements between bound dyes e.g. adjacent intercalated and groove bound dyes forming adventitious dimers. If two monomers are stacked in a twisted arrangement in a dimer, this gives rise to exciton splitting showing equal but opposite lobes in the CD spectrum centered on the absorption maximum. If additional dye molecules stack on the dimer, the splitting pattern depends on the magnitude and helical sense of the twist angle between adjacent TMs (Figure 3). For example, the DNA splitting pattern at 260 nm arising from stacked bases is $+/-$ with increasing energy: this is consistent with a right-handed helical arrangement of TMs, as expected for B-DNA.

RESULTS

Methylene Blue (MB)

(a) *Aqueous Solution.* The monomeric absorption spectrum of MB (5 μM) in the visible region has the expected maximum at 664 nm with a vibronic shoulder at ca. 615 nm (Figure 4, left). At 67 μM , the 664 nm peak is attenuated, and a distinct peak is observed at 610 nm accompanied by weak absorption about 715 nm that is attributed to MB dimer.⁸⁻¹⁰ The dimer peak is observed at both low (5 mM phosphate) and high (200 mM phosphate) ionic strength, and makes a relatively larger contribution in the latter case where electrostatic repulsion between dyes is attenuated. Notably, UV absorption is not significantly perturbed by dimer formation.

Figure 4. Spectra of MB [left] and Th [right] in aqueous solution (67 μM) at low (5 mM phosphate) and high (200 mM phosphate) ionic strength, and scaled monomeric spectra of MB and Th (5 μM).

(b) *[Poly(dG-dC)]₂* Absorption spectra (Figure 5) show that binding to [poly(dG-dC)]₂ at P/D=3 disrupts MB dimerization. In 200 mM phosphate, and more so in 5 mM phosphate, the dimer/monomer absorption ratio decreases compared to solution.

Figure 5. Absorption, LD and CD spectra of MB (67 μ M) with (top) [poly(dG-dC)]₂ and (bottom) [poly(dA-dT)]₂ at P/D = 3 in low (5 mM phosphate) and high (200 mM phosphate) ionic strength.

The UV/vis spectra cannot distinguish between species that are bound to DNA and those which remain free in solution. Multiple equilibria likely exist between free monomeric dye, intercalated monomer, externally bound monomer, free dimeric/aggregated dye, and externally

bound dimer/aggregates. To clarify which species are tightly associated with DNA, we turn to the polarized spectroscopy techniques of linear dichroism and circular dichroism.

In 200 mM phosphate, the negative LD spectral profile is very similar to that of intercalated monomeric MB across the entire spectral range which suggests that dimers have a low affinity for the polyanion at high ionic strength. Nonetheless, some bound dimers must be present that do not contribute a significant LD signal (e.g. oriented at 54.7°) since the CD at 615 nm is more negative than that of purely intercalated dye (notably, the CD of intercalated dye is unaffected by IS). Since no exciton splitting occurs, this signal likely represents an induced CD signal for a bound dimer within which the two dyes are arranged non-chirally.

(c) $[poly(dA-dT)]_2$ This polynucleotide shows a greater ability to induce extensive self-assembly of MB than $[poly(dG-dC)]_2$. In the absorption spectra at both low and high ionic strength (Figure 5), the monomer and dimer peaks are attenuated as a shorter wavelength band (565 nm) becomes apparent at a wavelength previously assigned to trimer.^{10,11,14}

In 5 mM phosphate, the LD spectrum shows a peak for monomer (664 nm) and a large peak for trimer (565 nm) with a dimer (605 nm) signal as a shoulder, and a signal beyond 700 nm. The CD spectrum shows exciton splitting centered near the trimer absorption maximum with the $+/-$ pattern of a right-handed helix, and a small positive CD is observed at long wavelength. The LD spectrum in 200 mM phosphate is unusual with a small negative signal for the monomer band, similar to intercalated dye LD, but a positive signal for the trimer band. This indicates that the dyes in the trimer at high ionic strength are oriented on average with their long axes $<54.7^\circ$ to the helix axis, different to the configuration at low salt. None-the-less, the CD exciton splitting in at for this signal remains right-handed.

(d) *Calf-Thymus DNA* The spectra for DNA at P/D=3 (ESI, Figure S1) are different from those with either alternating polynucleotide, demonstrating that the sequence-dependence of stacking produces heterogeneity of binding. In 5 mM phosphate buffer, strong aggregation is observed in absorbance, LD and CD, similar to [poly(dA-dT)]₂. A large $-/+$ exciton splitting is observed in the dimer band, but the spectrum is likely a convolution of signals from several species. Aggregation is reduced in 200 mM phosphate and the LD spectrum is more similar to that with [poly(dG-dC)]₂, but with an additional trimer band. The CD spectrum in 200 mM phosphate appears to represent a splitting about the dimer maximum absorbance convoluted with induced CD for intercalated dye. These results with DNA are very similar to those previously reported by Nordén and Tjerneld³¹ for slightly different concentrations which were interpreted in terms of "accidental" dimerization of a groove-bound dye with an intercalated dye in a left-handed configuration.

Thionine (Th)

(a) *Aqueous Solution* The absorption maximum of monomeric Th is at higher energy (598 nm) than MB and the vibronic shoulder is much less prominent (Figure 4). At 67 μ M Th there is evidence for formation of dimers in water at a wavelength (565 nm) coinciding with the vibronic shoulder. Increased ionic strength had insignificant influence on the spectra.

(b) [poly(dG-dC)]₂ At both ionic strengths, the absorption spectra are slightly red-shifted compared to solution and a new shoulder is observed at 525 nm which is assigned to aggregates (Figure 6). In 200 mM phosphate, additional shoulders occur at 480 nm and 670 nm. In 5 mM phosphate, more dimer is present than in solution; in 200 mM phosphate, less dimer is present than in solution.

Figure 6. Absorption, LD and CD spectra of Th (67 μM) with (top) [poly(dG-dC)]₂ and (bottom) [poly(dA-dT)]₂ at P/D = 3 in low (5 mM phosphate) and high (200 mM phosphate) ionic strength.

The LD spectrum in 5 mM phosphate has high negative intensity and mirrors the absorption spectrum. Exciton splitting (+/-) at 530 nm in CD is consistent with formation of a right-handed aggregate. The positive CD peak that is observed near 630 nm is found for all bound Th spectra,

including pure intercalation. In 200 mM phosphate, the LD spectrum resembles that of intercalated Th with superimposed positive LD for the 480 nm band and negative LD for the 670 nm band. The strongest CD feature is the large right-handed exciton splitting (+/–) around 520 nm which is asymmetric, probably due to a convolved (+/–) splitting at 480 nm. The remaining CD signal resembles intercalated Th.

(c) *[poly(dA-dT)]₂* Binding to this polynucleotide induces changes in the absorption spectrum consistent with increased formation of dimers (560 nm), particularly in 5 mM phosphate (Figure 6). A shoulder at 522 nm and increased absorption at $\lambda > 650$ nm is also observed, consistent with formation of higher order aggregates. However, the dimer bands in 5 mM phosphate are not as prominent in LD as in absorption, and they are not apparent in 200 mM phosphate where the spectrum is similar to intercalated dye. Nonetheless, the presence of dimers at both ionic strengths is apparent in the CD spectra where (+/–) right-handed exciton splitting is observed about 550 nm at both high and low salt. This indicates that bound dimers either have a random orientation or have a fixed orientation that is less perpendicular than intercalated dye. Positive CD is observed at 630 nm for all samples, and unusual negative CD is observed at $\lambda > 650$ nm for the P/D = 3 samples. The spectra are complicated and appear to arise from convolution of signals from dyes bound in several modes.

(d) *Calf-Thymus DNA* At P/D 3, binding to DNA in 5 mM phosphate causes a red-shift in the monomer absorption and an increased dimer peak relative to the monomer peak. The visible LD spectrum is negative but the shape is different from the absorption spectrum, with no contribution from the dimer and resembles that of intercalated monomer. The CD spectrum does not show the dimer or aggregated exciton splitting that is observed with the polynucleotides, but neither is it identical to the intercalated dye spectrum.

In 200 mM phosphate, the absorption spectrum resembles that of intercalated monomer with no indication of dimers. However, there are small shoulders at 480 nm and 670 nm as observed with [poly(dG-dC)]₂. The LD of this sample is also similar to that observed with [poly(dG-dC)]₂ and the spectrum resembles that of intercalated dye with an additional negative peak at 670 nm and a positive peak at 480 nm. The CD spectrum has similar features to intercalated dye with low intensity (+/-) splitting about the absorption maxima at 670 nm and 480 nm which cannot be deconvoluted in the polynucleotide spectra.

Azure B (AB)

The aqueous absorption spectrum of AB is similar in shape to MB with a maximum at 646 nm and a distinctive dimer band at 595 nm (Figure 7). Spectra were recorded for AB with [poly(dA-dT)]₂ and [poly(dG-dC)]₂ in high salt conditions where the free dye absorption spectrum shows strong dimer formation. This dye was studied to establish the extent to which a single exocyclic N-H influences the stacking interactions with DNA.

Figure 7. Spectra of Azure B (AB) with [poly(dA-dT)]₂ and [poly(dG-dC)]₂ at high binding ratio. [AB] = 67 μ M; [base] = 200 μ M; buffer = 200 mM phosphate (pH 6.9).

With [poly(dG-dC)]₂, monomer and dimer absorption bands are diminished, and a blue-shifted peak appears at 545 nm with a shoulder at 570 nm. The 570 nm band is assigned to trimer, and the 545 nm peak to a higher order aggregate. The negative LD spectrum mirrors the absorption in the monomer and dimer regions and has a very intense band for aggregate 545 nm with trimer shoulder. Very large exciton splitting at 545 nm is observed in the CD spectrum, with a (–/+) pattern indicative of a left-handed aggregate. The symmetry of the exciton band is broken at higher wavelength, with negative shoulders in the dimer and monomer absorption wavelengths indicating that lower order species with intrinsic or induced chirality are also present, although their spectra are not easily deconvoluted.

With [poly(dA-dT)]₂, the dimer/monomer absorption ratio is reduced and a small shoulder near 570 nm but no aggregated band is observed. Negative LD signals are observed in the monomer and dimer bands, but LD_M/LD_D is lower than observed in absorption indicating that some dimers remain free in solution or produce no LD signal when bound. The CD spectrum shows symmetric splitting (+/–) at 575 nm even though there is no substantial absorption or LD signal at this wavelength. We assign this to trimer and the splitting is consistent with a right-handed helical arrangement.

DISCUSSION

The formation of MB and Th dimers in solution is well known from studies of absorption spectra variations as a function of concentration or ionic strength.⁸⁻¹³ For phenothiazine dyes, the transition moments (TMs) of their visible absorption bands are polarized along the long axis of the tricyclic system.³³ The dimers are commonly described as cofacial stacks with parallel TMs (Figure 2), a configuration that is expected to result in a high intensity blue-shifted absorption peak (H-band), with a low intensity peak to the red of the monomer maximum (Figure 3). At

very high dye concentrations¹⁰⁻¹³ or when the dyes are adsorbed at interfaces,¹⁴⁻¹⁸ increasingly blue shifted absorption bands are observed arising from higher order cofacial aggregates. In this work we observe similar stacking behavior when the dyes are associated with double-stranded B-DNA. Aspects of phenothiazine dye aggregation with DNA at low P/D and high dye concentration, monitored by changes in UV/vis absorption, LD and CD spectra, have been previously reported.^{20-22,30,31,33} However, few studies have compared different dyes or different DNAs. The studies described here provide a wide overview of how stacking depends on nucleic acid sequence and on small variations in dye structure, as well as the ionic strength of the environment. Binding models are constructed by amalgamating the complementary information provided by UV/vis, LD and CD spectra.

Effect of Dye Exocyclic Amino Methylation

Our findings show a remarkable influence of exocyclic amino methylation on stacking of phenothiazine dyes with DNA. MB, which is fully methylated on the exocyclic amines, readily aggregates with [poly(dA-dT)]₂ irrespective of background salt, but the orientation of the aggregates changes dramatically with increasing ionic strength. By contrast, MB dimerization is reduced in the presence of [poly(dG-dC)]₂, although bound dimers are found at low ionic strength. However, these are largely removed at high ionic strength where bound MB appears to be predominantly intercalated. Thionine has fully de-methylated exocyclic amines and shows increased aggregation with both [poly(dA-dT)]₂ and [poly(dG-dC)]₂. Higher order aggregation and dimers are observed with [poly(dA-dT)]₂ at low ionic strength, but only dimers remain when ionic strength is increased. With [poly(dG-dC)]₂, higher order aggregate is present without dimer in both 5 mM and 200 mM phosphate but an orientation change occurs as the ionic strength increases. The behavior of AB with one demethylated group differs from that of the other dyes.

With [poly(dG-dC)]₂, AB produces a highly oriented aggregate that has different LD and CD spectra than those of either MB or Th. With [poly(dA-dT)]₂, the behavior is quite similar to that of Th with only dimers observed. This demonstrates how a small change at the extremity of the dye structure has a large effect on dye-dye interactions at the surface of a DNA molecule. Since dimerization of phenothiazines in water is not greatly affected by structure,⁸ the variations must be driven by differences in the external binding of the dyes to DNA.

Effect of DNA Sequence

The polynucleotide sequence influences stacking but in a variable manner. [Poly(dA-dT)]₂ induces substantially higher order stacking of MB than does [poly(dG-dC)]₂. Th and AB each demonstrate more stacking with [poly(dG-dC)]₂. Since stacking interactions are possible for all three dyes with both polynucleotides, this suggests that the deep minor groove of AT-rich sequences is not required to promote aggregation of phenothiazines. This differs from the requirements for DNA-templated assembly of helical cyanine dye H-aggregates, as reported by Armitage and others,⁵⁰⁻⁵³ where 3,3'-diethylthiadicyanin dimers line up along the minor groove of [poly(dA-dT)]₂ or [poly(dI-dC)]₂ but cannot be accommodated in the shallower minor groove of [poly(dG-dC)]₂. Effects of DNA sequence on Hoechst aggregation have previously been reported by Rodger and coworkers.⁵⁴

Effect of Ionic Strength

In aqueous solution, dimerization and high order aggregation of planar cationic dyes such as phenothiazines is enhanced as the ionic strength increases, due to reduction in electrostatic repulsion with high concentrations of background anions. DNA binding of phenothiazines is an electrostatically attractive process, so the association of phenothiazines at low P/D is reduced when ionic strength increases.³⁰⁻³² Stacking on DNA is typically of much higher order than

observed in solution since inter-dye repulsion is attenuated in the negative electric field of DNA. At higher ionic strength, when there is less attraction of dyes to DNA, dye-dye repulsion becomes more important and stacking is generally reduced.

However, for some systems, dye aggregation is not reduced with increasing ionic strength, but the structure of the aggregate changes. For MB with [poly(dA-dT)]₂, the trimer LD changes from negative to positive as ionic strength increases. This indicates that the dyes at high ionic strength are oriented with their long axes <54.7° to the helix axis, different to the more perpendicular configuration at low salt. Nonetheless, the CD exciton splitting for this signal remains right-handed. It should be noted that the binding of MB to [poly(dA-dT)]₂ has an unusual ionic strength dependence.³⁰ At low salt, intercalation is favored but with increasing ionic strength there is a tendency for a high proportion of MB monomers to occupy groove-bound sites. We suggest that these groove-bound monomers could provide templates for an alternative stacking pattern of MB with [poly(dA-dT)]₂ at high salt.

For Th with [poly(dG-dC)]₂ and DNA at high ionic strength, a new positive LD appears at 480 nm and a new negative LD at 670 nm. These bands are apparent only for P/D ≤ 3 (Figure S4) and are presumably due to highly ordered aggregates close to condensation. At P/D < 1 precipitation occurs resulting in an inverted LD spectrum at these wavelengths.

Chirality of the Aggregates

For many of the samples where LD shows bound dimers or aggregates, exciton splitting is observed in the CD spectra. Since the splitting patterns are centered on the multimer absorbances, this indicates interactions between bound dimers or more aggregated dyes rather than simply between stacked monomers. For Th with both [poly(dA-dT)]₂ and [poly(dG-dC)]₂ at high and low ionic strength, the lowest wavelength splitting in the visible (+/–) is consistent with

right-handed chirality of the dimers or aggregates. The splitting pattern is overlaid on the induced CD spectrum of intercalated Th, as seen in Figure S3 (ESI) which shows that aggregation depends on total dye concentration as well as P/D. The CD spectra with DNA show no distinct splitting patterns, so chirality cannot readily be analyzed. MB with [poly(dA-dT)]₂ also shows right-handed chirality of aggregates at both ionic strengths. However, with [poly(dG-dC)]₂, left-handed splitting is observed in the dimer band, and this is also the case with DNA at both ionic strengths. AB likewise shows right-handed stacking (dimer) with [poly(dA-dT)]₂, but left-handed stacking (aggregate) with [poly(dG-dC)]₂.

Structural Interpretation

Strong UV signals from the phenothiazine dyes interfere with interpretation of the LD and CD spectra of DNA, but no dramatic changes occur to indicate that the DNA conformation is perturbed by formation of dye aggregates. For each sample of MB, Th, and AB, negative LD signals at the wavelengths for bound monomer suggests that intercalated dye is always present in addition to externally bound dye. The LD, and sometimes CD, signals for multimeric dye structures are overlaid on the monomer signals, as well as on each other. In addition to dimerization and stacking of dyes on DNA which have distinctive spectroscopic signatures, additional signals can arise from adventitious interactions between intercalated and externally bound dyes. From amongst the composite information contained in the recorded spectra for all these species, it can be difficult to extract definitive structural information. Molecular dynamics studies⁵⁵⁻⁵⁸ have concluded that MB has a low energy binding mode in the minor groove of [poly(dA-dT)]₂, with the exocyclic amines oriented into the groove, consistent with LD and CD findings of mixed intercalation/groove binding at high salt.³⁰ LD and CD studies show that this effect is much less significant for Th.³² Noguiera⁵⁸ recently reported that MB groove binding is

also relatively stable with $[\text{poly}(\text{dG-dC})]_2$ and is stabilized by a hydrogen bond when the amino groups face away from the groove, since the inward orientation is sterically unfavorable. From LD, we find that intercalation is always observed for both MB and Th with $[\text{poly}(\text{dG-dC})]_2$ at high P/D.^{30,32} Nonetheless, at lower P/D when intercalation sites are filled, additional minor-groove binding can occur.

Figure 8. Potential binding models for stacking of phenothiazine dyes on DNA.

Figure 8 shows some putative modes of assembly, illustrated for minor groove occupation although we cannot exclude the possibility that the aggregated dyes occupy the major groove. Whilst CD is extremely sensitive to the organization of bound dyes, and to dye-dye interactions, it is difficult to interpret the spectra in terms of defined structures. Even though absolute structures cannot be determined, the sign and magnitude of the LD spectra as well as the helical sense of the CD signals contain structural information, allowing different aggregate configurations to be permitted or excluded. In most cases, H-aggregates with right-handed coupling combined with negative LD are observed, which can be interpreted as stacking that follows the screw-sense of the DNA helix, either by stacking along a groove (**IV**) or along the backbone (**III**). However, considering the strength of intercalation and the likelihood that all

intercalation sites are filled before external binding occurs,³¹ the right-handed adventitious dimer configuration (**IX**) is likely to be a major contributor to the spectra. Indeed, nearest-neighbor coupling between such dimers could produce CD splitting in the dimer absorption region. In order to demonstrate H- rather than J-aggregate splitting, the center-to-center displacement of stacked dyes must be less than half the length of the transition moment. Therefore, we note that monomeric (**VI**) or dimeric (**VII**) dye binding in the minor groove in an end-to-end arrangement would produce a J-aggregate with right-handed splitting and positive LD.

For MB with [poly(dG-dC)]₂ and DNA at low ionic strength, and for AB with [poly(dG-dC)]₂ at high ionic strength, left-handed splitting is observed in the dimer band with strong negative LD. Such signals could potentially be explained by dimers in configuration **I** in Figure 8, although it would be sterically demanding for the dyes to remain at <54.7° to the helix axis, and an extended assembly would be more likely to adopt configuration **II**. Instead, the configuration (**VIII**) previously proposed by Nordén and Tjerneld for MB with DNA at low ionic strength³³ is a more feasible model. Thus we assign that configuration also to MB and AB with [poly(dG-dC)]₂. Notably, it is only observed for dyes with amino-methyl substituents and for sequences containing GC base-pairs. For MB with [poly(dA-dT)]₂ at high IS, positive LD for a right-handed H-aggregate is seen for the trimer absorption, while monomer and dimer bands retain negative LD. Such signals are consistent with the arrangement in model **V**, in either the minor or major groove, so that the MB transition moment is oriented at >54.7° to the helix axis. This is not seen with AB since trimers are not formed under these conditions, so no commonality is conferred by the amino-methyl substituents for interaction with [poly(dA-dT)]₂. For Th with [poly(dG-dC)]₂ at high IS, very strong LD is observed at short (positive LD) and long (negative LD) wavelengths where there is no obvious absorbance and no significant contribution to CD.

We assign this to a pre-condensation aggregate with strong LD due to high orientability. On precipitation, LD at the same wavelengths is observed but with inverted sign.

These results may be compared with reports on the aggregation of other groups of chromophores with DNA. Armitage and coworkers⁵³ found that monocationic 3,3'-diethylthiadicarbocyanine forms regular right-handed H-aggregates of dimers in the minor groove of [poly(dA-dT)]₂ or [poly(dI-dC)]₂.⁵⁰ The tricationic dye 3,3'-dimethyl(trimethylammonium)thiadicarbocyanine formed a J-aggregate with [poly(dI-dC)]₂ at low temperature but a H-aggregate at high temperature.⁵¹⁻⁵² However, unlike the phenothiazines, neither of these dyes showed aggregation in the presence of [poly(dG-dC)]₂. Porphyrins, on the other hand, stack with both [poly(dA-dT)]₂ and [poly(dG-dC)]₂ to form external helical aggregates,⁵⁹⁻⁶¹ which are modulated by the charge and structure of the molecules. Hoechst binds in the AT minor groove at high P/D but at low P/D is observed to form different dimer and aggregate forms depending on sequence.^{26,54} Some of these dyes intercalate as well as bind externally, like the phenothiazines, but many demonstrate DNA-induced dimerization and aggregation at higher P/Ds than those used in our study and at concentrations where dimerization is not observed in solution. By considering phenothiazine aggregation in the context of other dyes behaviors, it is clear that a wide diversity of non-intercalative interactions are possible which depend on dye structure, P/D ratio, DNA sequence, and ionic strength.

CONCLUSION

In summary, this study reveals that the aggregation of phenothiazine dyes with polydeoxynucleotides at low P/D ratios has a diversity of structure that depends on DNA sequence, extent of methyl substitution on the exocyclic amines, and the ionic strength. At P/D=3, the intercalation sites are saturated and excess dye binds in the grooves or by association

with the backbone, forming dimers, trimers, and possibly higher aggregates. Most stacked dyes are observed to be right-handed H-aggregates with negative LD consistent with stepped stacking in the minor groove or along the backbone, or with adventitious dimers between intercalated dye and minor groove bound dye. The features of MB and AB with [poly(dG-dC)]₂ at low ionic strength are explained by left-handed dimers between an intercalated dye and a dye bridging the minor groove. MB with [poly(dA-dT)]₂ at high IS gives unusual positive LD spectra consistent with aggregates that bridge the grooves. Finally, Th with [poly(dG-dC)]₂ at high IS gives pre-condensation spectra typical of highly aggregated species. In general, aggregation of Th and AB are enhanced with [poly(dG-dC)]₂ whilst aggregation of MB is enhanced with [poly(dA-dT)]₂. In general, AB behaves in a manner that is different from either other dye. Despite MB, AB, and Th possessing a common tricyclic framework, the organization of their polynucleotide-facilitated aggregates depends sensitively on the extent of methylation of the exocyclic amines.

Supporting Information. Absorption, LD and CD spectra with calf-thymus DNA – CD spectra for thionine with [poly(dG-dC)]₂ at fixed P/D as a function of concentration of both thionine and polynucleotide – LD spectra for thionine with [poly(dG-dC)]₂ as a function of thionine concentration at constant polynucleotide concentration – predictions of CD spectra for dyes stacked along a groove.

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Author Contributions

The experiments were performed by EMT. The manuscript was written through contributions of EMT and BN. Both authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

AB, azure B; CD, circular dichroism; LD, linear dichroism; MB, methylene blue; [poly(dA-dT)]₂, polydeoxyadenylic-thymidylic acid; [poly(dG-dC)]₂, polydeoxyguanylic-cytidylic acid; Th, thionine.

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Table of Contents Graphic

Structural Heterogeneity in Polynucleotide-Facilitated Assembly of Phenothiazine Dyes

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SUPPORTING INFORMATION

Figure S1. Absorption, LD, and CD spectra for MB with CT-DNA. [MB] = 67 μ M. P/D = 3. Low ionic strength = 5 mM phosphate (pH 6.9); high ionic strength = 200 mM phosphate (pH 6.9). The intercalated dye spectrum is for a high P/D as previously reported [E. Tuite and B. Nordén, *J. Am. Chem. Soc.*, 1994, **116**, 7548]

Figure S2. Absorption, LD, and CD spectra for Th with CT-DNA. [Th] = 67 μ M. P/D = 3. Low ionic strength = 5 mM phosphate (pH 6.9); high ionic strength = 200 mM phosphate (pH 6.9). The intercalated dye spectrum is for a high P/D as previously reported [E. Tuite and B. Nordén, *Spectrochim. Acta A*, 2018, **189**, 86-92]

Figure S3. CD spectra for Th with [poly(dG-dC)]₂. P/D = 3. Varying Th and [poly(dG-dC)]₂ concentrations, as indicated in the legend in μM units. Buffer = 5 mM phosphate (pH 6.9).

Figure S4. LD spectra for Th with $[\text{poly(dG-dC)}]_2$. $[\text{GC}] = 200 \mu\text{M}$. Varying $[\text{Th}]$. P/D indicated in the legend. Buffer = 5 mM phosphate (pH 6.9).

Figure S5. Predicted CD spectra for different organisations of dye stacked along the minor groove.