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Minor-Groove Binding Drugs: Where Is the Second Hoechst 33258 Molecule?

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Supporting Information

ABSTRACT: Hoechst 33258 binds with high affinity into the minor groove of AT-rich sequences of double-helical DNA. Despite extensive studies of this and analogous DNA binding molecules, there still remains uncertainty concerning the interactions when multiple ligand molecules are accommodated within close distance. Albeit not of direct concern for most biomedical applications, which are at low drug concentrations, interaction studies for higher drug binding are important as they can give fundamental insight into binding mechanisms and specificity, including drug self-stacking interactions that can provide base-sequence specificity. Using circular dichroism (CD), isothermal titration calorimetry (ITC), and proton nuclear magnetic resonance (^{1}H NMR), we examine the binding of Hoechst 33258 to three oligonucleotide duplexes containing AT regions of different lengths: $[d(CGCGAATTCGCG)]_2$ (A_2T_2), $[d(CGCAAATTTGCG)]_2$ (A_3T_3), and $[d(CGAAAATTTTCG)]_2$ (A_4T_4). We find similar binding geometries in the minor groove for all oligonucleotides when the ligand-to-duplex ratio is less than 1:1. At higher ratios, a second ligand can be accommodated in the minor groove of A_4T_4 but not A_2T_2 or A_3T_3. We conclude that the binding of the second Hoechst to A_4T_4 is not cooperative and that the molecules are sitting with a small separation apart, one after the other, and not in a sandwich structure as previously proposed.

INTRODUCTION

Drugs that target DNA with high sequence specificity have gained increasing attention for their potential medicinal applications. These include molecules that can control gene expression by binding to only specific genes and there interact and inhibit DNA-binding proteins such as transcription factors. The DNA binding molecules of interest vary from small low-molecular-weight compounds to large polypeptides or synthetic protein complexes.[1−7] Particularly interesting are the oligoamide drugs developed by Dervan,[8] which bind sequence-specifically in the minor groove of DNA by forming back-folded hairpin structures that, due to their asymmetric sandwich stacking, can discriminate between the two walls of the minor groove, that is, between A-T and T-A base pairs. Examples of larger constructs are the zinc-finger small protein motifs developed by Klug[4] for gene correction therapy.

Low-molecular compounds have the advantage that they can generally more easily pass the cellular membranes and faster reach the DNA in the cell nucleus, and their molecular structure can also be manipulated relatively easily to alter both binding and membrane-translocation properties. One important class of low-molecular weight compounds is represented by Hoechst 33258, a bis(benzimidazole) derivative that has been shown to display antitumor and antibiotic properties.[9−12] It fluoresces brightly when bound to double-stranded DNA but is optically silent (quenched) when free in buffer solution, and it is therefore used for quantification of DNA and as a chromosomal stain in microscope imaging.[13−15] The dye binds strongly in the minor groove of DNA sequences that are rich in adenines and thymines.[3,16] This minor-groove binding is interesting since it is strongly endothermic, and thus entropically driven, in contrast to intercalation, for which the enthalpic contribution usually dominates.[17−20] When a ligand is bound in the DNA minor groove, it can interact with minor-groove binding proteins needed for gene expression. Gene inhibition has been documented for Hoechst 33258 as well as for other minor-groove binding molecules, through their interference with TATA-box transcription factors.[12]

Hoechst 33258, together with a few other minor-groove binders, such as distamycin and netropsin, is one of the model compounds for designing new DNA-binding drugs.[1,11] Its structure is typical for a minor-groove binder (Figure 1): the molecule is crescent-shaped and consists of flat hydrophobic aromatic rings, creating a snug fit between the rather hydrophobic sugar walls in the minor groove. Hoechst 33258 is positively charged, matching the strongly electronegative potential at the floor of the minor groove of AT regions,[21] which is more negative than regions with high GC content. It

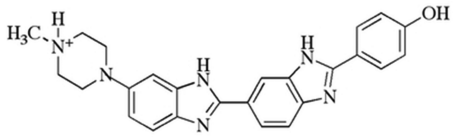

Figure 1. Structure of Hoechst 33258 (neutral pH).

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also contains several potential hydrogen-bond donors and acceptors that can interact with suitably positioned hydrogen-bond acceptors and donors of adenine and thymine in the minor groove.[16,21,22]

However, there are some peculiarities in the DNA binding of Hoechst 33258. Despite clearly strong electrostatic interactions, including hydrogen-bonding and dipolar interactions, there is compelling evidence indicating that it is in fact the hydrophobic interactions in the narrow minor groove of AT sequences that are a major contributing factor for the preferential binding there.[23,24] DNA sequences with high AT content have a narrower groove width (3−4 Å) than the minor grooves of mixed DNA sequences (6 Å),[25] allowing a snug fit of the molecule, while GC-rich sequences display an even wider groove width than the latter.[3] Further supporting the structural attractiveness of the DNA minor groove of AT-DNA, its floor is smooth and convex, while the floor of GC sequences is interrupted by the exocyclic amino groups of guanines protruding into the groove and obstructing good fit of a minor groove-binder. It has been shown by quantitative DNase I footprinting studies that Hoechst 33258 is more sensitive to changes in AT sequence composition than netropsin and distamycin[26] and that it can bind with nanomolar affinity to certain AT-rich sequences.[1,27] A striking example is found when the sequence of adenines and thymines is alternated in oligomers: fluorescence titrations and footprinting studies clearly show that it has some 170 times higher affinity for 5′-AATT-3′ than for 5′-TTAA-3′.[26,28,29]

Binding kinetics of Hoechst 33258 to DNA is not fully understood either, and multiple binding modes of the dye to AT-rich DNA have been suggested.[28,30,31] Such apparent multiple binding modes might indicate that the dye binds at several locations and/or orientations in the AT sequences,[28] a hypothesis supported by the occurrence of a variety of crystal structures of Hoechst−DNA complexes.[22,32−37] The crystal structures report variations regarding what base pairs Hoechst 33258 is bridging in the AT sequence, and also in which polar orientation along the groove the molecule is pointing. However, all structures consistently confirm that Hoechst 33258 covers about four consecutive AT base pairs, while the polar orientation may vary. In crystal structures where Hoechst 33258 binds to oligonucleotides containing 5′-GAATTC-3′ in the middle, the dye has been reported to cover AATT, ATTC, or GAAT, with the phenyl group usually facing the narrow AT tract.[22,32−34,37] Also, a high-resolution NMR structure has shown that Hoechst 33258 behaves similarly in solution: it covers exactly the AATT in such an oligonucleotide.[21] Crystal structures of oligomers containing a longer AT sequence, 5′-CAAATTTG-3′, show that the dye covers ATTTG or AATTT, again with the phenyl group pointing toward the middle of the AT sequence.[35,36] When the AT region is expanded even further, to 5′-CAAAATTTTG-3′, two spectroscopic studies suggest that only one dye binds in the middle of the oligomer,[31,38] while yet another suggests that Hoechst 33258 can bind as a monomer, dimer, or tetramer to 5′-CAAAATTTTG-3′.[39] Loontiens et al.[30] suggest that the drug may even form larger aggregate complexes, up to six dye molecules per five base pairs, when the AT sequence is expanded to a $[poly(dAdT)]_2$ duplex. They propose that the binding of the first molecule occurs in the minor groove, as described previously, but that the subsequent molecule binds as a sandwich on top of the first dimer, in a head-to-tail fashion. Further binding modes have been reported in pure GC contexts, where a (weaker) binding by intercalation was early detected.[40,41] In conclusion, despite many attempts to characterize the structures of minor-groove-binding drug molecules, represented by Hoechst 33258, no clear generic picture of their interaction has appeared. In particular, the question how additional binding of the drug to the minor groove may occur appears to be not satisfactorily answered.

To get a more detailed understanding for how a potential second Hoechst 33258 molecule binds to A_nT_n sequences, we have performed a systematic spectroscopic and thermodynamic study of the binding to DNA duplex oligonucleotides with AT sequences of varied and increasing lengths: 5′-CGCGAATTCGCG-3′ (A_2T_2), 5′-CGCAAATTTGCG-3′ (A_3T_3), and 5′-CGAAAATTTTCG-3′ (A_4T_4). The spectroscopic data have given a considerably improved picture of the structure of the complex in solution, while the thermodynamic analysis provides information about the nature of binding interactions. The data received from the two different experimental methods have been analyzed in a combined data set. Additionally, we have also analyzed the Hoechst−A_4T_4 complex by ^{1}H NMR to obtain further details about the interactions of the complex in solution.

Using isothermal titration calorimetry (ITC) and circular dichroism (CD), we find that the binding of Hoechst 33258 to A_2T_2 and A_3T_3 is very similar, only a single dye molecule binds into the DNA minor groove. When the AT-site is saturated, further increased concentration of Hoechst 33258 results most probably in a nonspecific electrostatic binding to the negatively charged DNA phosphate backbone.[42,43] However, in contrast to some conclusions previously published,[30,31,38,39] we observe that for the oligonucleotide with the longer AT tract, A_4T_4, a second Hoechst 33258 molecule can also be accommodated in the minor groove.

Furthermore, the two modes of binding are clearly not cooperative, meaning that the ratio of their stepwise binding constants $K_1/K_2 \gg 1$, thus the first site is filled before binding to the second site commences. We conclude from quantitative analysis of spectroscopic and thermodynamic data that when two drug molecules bind to A_4T_4, the dye molecules prefer not to be stacked on top of each other (sandwiched) or to sitting next to each other in the minor groove in such a way that they are partly overlapping each other (contiguous binding), but instead they bind in the minor groove following each other with a small distance apart. Interestingly, the binding of a single Hoechst 33258 molecule to A_3T_3 and A_4T_4 shows enthalpic variations that could only be fitted together with the CD data by global analysis when two thermodynamically different populations of DNA were utilized. To explain such variations, which have been previously observed by others,[44,45] one group put forward a hypothesis that DNA may occur in two "populations" representing stable but different states of hydration. However, our NMR data do not indicate a significant heterogeneity, and we are instead inclined to believe that the enthalpic phenomena have a kinetic origin.

EXPERIMENTAL SECTION

Circular dichroism (CD) and isothermal titration calorimetry (ITC) experiments were performed in 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid; Sigma], pH 6.2, and 200 mM NaCl. Hoechst 33258 (Aldrich) was quantified in aqueous solution spectroscopically by use of ε_{338} = 42 000 $M^{-1}\cdot cm^{-1}$, and oligomers were purchased from ATDBio, purified by HPLC. The oligomers were annealed by heating to 90 °C

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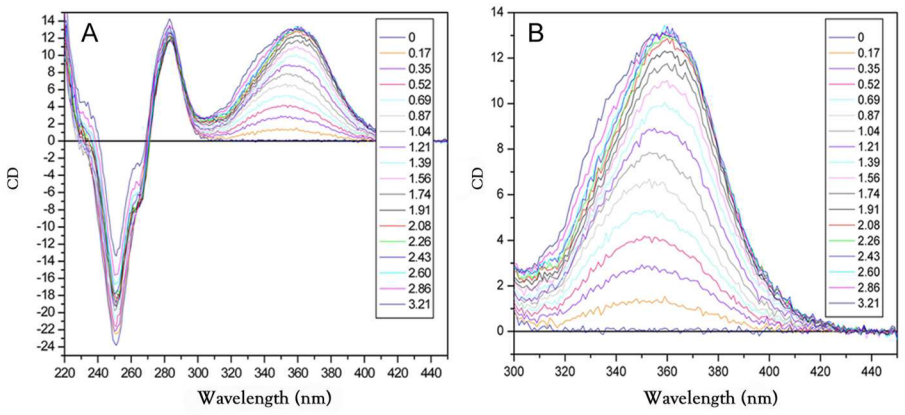

Figure 2. Raw CD spectra of Hoechst 33258 titrated into A_4T_4. CD signals are normalized to DNA concentration. (A) Whole spectrum; (B) close-up of induced CD upon binding of Hoechst 33258 to DNA. Hoechst 33258 (300 μM) is titrated into 5 μM A_4T_4 duplex with stoichiometric ratios of drug molecule per oligomer. For more spectra, see Supporting Information.

followed by slow cooling to 5.5 °C over 6 h, and the concentrations were determined by use of ε_{258} = 193 070 $M^{-1}\cdot cm^{-1}$ for d(CGCGAATTCGCG)$_2$ (A_2T_2), ε_{258} = 187 077 $M^{-1}\cdot cm^{-1}$ for d(CGCAAATTTGCG)$_2$ (A_3T_3), and ε_{259} = 185 874 $M^{-1}\cdot cm^{-1}$ for d(CGAAAATTTTCG)$_2$ (A_4T_4). For ITC measurements, the oligomers were dialyzed for 48 h against the same buffer with a 3500−5000 MW cut off. Hoechst 33258 was then dissolved in the dialyzed buffer. The mixing ratio is defined as the total number of Hoechst 33258 molecules relative to the number of oligomer duplex ([Hoechst 33258]/[oligomer duplex]). For the NMR measurements, A_4T_4 was annealed by heating to 50 °C, at which temperature the imino signals disappeared, followed by slow cooling to 25 °C (1 °C/min), where they reappeared. The buffer used in the NMR measurements was 20 mM deuterated Tris-HCl, pH 6.5.

Isothermal Titration Calorimetry. ITC measures the heat arising from a reaction, or interaction, where one reaction reactant is titrated in small aliquots into a cell containing the other reactant. The output is in the form of peaks, each corresponding to the heat that the instrument has to add or subtract in order to keep the sample cell and reference cell at the same constant temperature. A typical experiment was titration of 400 μM ligand in 3.5 μL aliquots at 180 s intervals into the sample cell, which contains 206 μL of 50 μM duplex solution (A_2T_2 and A_3T_3) or 25 μM duplex solution (A_4T_4). Calorimetric experiments were carried out on a MicroCal200 instrument (MicroCal).

Circular Dichroism. CD is defined as the differential absorption of circularly polarized light:

$$\mathrm{CD}(\lambda) = A_L(\lambda) - A_R(\lambda) \quad (1)$$

where A_L and A_R are the absorbance of left- and right-polarized light, respectively. Hoechst 33258 is an achiral molecule and therefore does not give rise to a CD signal. However, when it is bound to DNA an induced CD can be detected, supposedly arising primarily from coupling with the chiral arrangement of bases in DNA. CD measurements were performed on a Chirascan instrument (Applied Photophysics). Typically 4 μL of 300 μM ligand was titrated into a 1 cm quartz cuvette containing 3 mL of 5 μM oligomer solution.

^{1}H NMR Titration. Spectra were recorded in Shigemi tubes with 200 μM DNA concentration in a 9:1 mixture of H_2O/D_2O. Hoechst 33258 was added in small amounts up to a final ligand-to-DNA ratio 2:1. The NMR spectra were recorded at 298 K on a Varian Inova 800 MHz spectrometer with Watergate solvent suppression.

Fitting of Theoretical Isotherms. The CD spectra from titrations of Hoechst 33258 into the three different oligomers (A_2T_2, A_3T_3, and A_4T_4) were examined by singular value decomposition (SVD) analysis in MatLab. A matrix **M** was constructed out of the CD spectra and factorized into three matrices **U**, **S**, and **V**, such that **U** and **V** are orthonormal, **S** is a non-negative diagonal matrix, and $\mathbf{M} = \mathbf{USV}^T$. The number of values in **S** that are distinctly above noise level can be assigned to the number of distinct species, or binding modes, in the ligand−DNA interaction. The corresponding columns in the **U** and **V** matrices are linear combinations of the CD spectra of the species and their concentration profiles, respectively. Thus, a theoretical binding isotherm is consistent with the experimental data only if it is contained in the subspace spanned by the significant **V** columns. A more detailed account of the fitting methods used can be found in Supporting Information.

RESULTS

Induced CD and UV−Vis Absorption. The CD spectrum of dsDNA arises mainly from exciton coupling between the $\pi \rightarrow \pi^*$ transition moments of the stacked DNA bases. This results in a bisignate feature centered at the absorption maximum of DNA at 260 nm, with a negative peak around 250 nm and a positive peak around 285 nm. Free Hoechst 33258, by contrast, being an achiral molecule, does not demonstrate any CD on its own. However, when it is bound to DNA it acquires an induced CD at 353 nm, an effect observed for all three oligomers at low binding ratios (CD spectra for Hoechst 33258 titrated into A_4T_4 are shown in Figure 2). The induced CD is positive, relatively strong, and slightly red-shifted compared to the absorption peak (at 338 nm) for the free drug chromophore, features that are in agreement with the ligand being positioned in the DNA minor groove, as previous studies of A_nT_n−Hoechst 33258 complex have also suggested.[1,16,22,33] The strong positive CD, consistent with binding in the minor groove, can be explained by nondegenerate exciton coupling with transitions in the nucleobases,[46] The CD spectra of the drug bound to A_2T_2 and A_3T_3 are very similar (Figure S1, Supporting Information), while the spectrum for the complex with A_4T_4 displays clear differences compared to those of the other duplexes. When the dye is titrated into a solution of either A_2T_2 or A_3T_3, the induced CD signal at 353 nm stops

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increasing when the ratio of Hoechst 33258 to oligomer duplex reaches 1:1, and after this point only a slowly increasing positive shoulder at 330 nm is detected. On the other hand, when Hoechst 33258 is titrated into A_4T_4, the induced CD signal first increases in similar manner as at 353 nm, but then it displays a distinct red shift at ratios above 1:1, from 353 toward 365 nm (Figure 2). This second red shift of the induced CD suggests that, after that the first binding site is completely filled, a second dye binds at another binding site. Qualitatively this suggests that the two modes of binding are not cooperative, contrasting the claim that Hoechst 33258 should bind cooperatively to $d(CTTTTCGAAAAG)_2$.[47] Thus, interestingly, we cannot see any evidence for cooperativity with our sequence $d(CGAAAATTTTCG)_2$. This variation is probably because the first Hoechst 33258 molecule is positioned in the middle of the A_4T_4 sequence (see NMR results below) and has to be pushed away off-center before the second molecule can enter the minor groove of the AT sequence. If the binding had been cooperative, the CD spectrum would consist of a mixture of signals arising from the two different binding sites at the same time, and the distinct red shift that we see at 1:1 would not have been observed.

The shape of the CD spectrum can also give information about how the two dye molecules sit relative to each other in the minor groove. The first Hoechst 33258 molecule obviously binds tightly in the minor groove of the AT sequence, having its long axis transition moment at an angle of near 45° (± 3°) relative to the helix axis of the DNA.[16,40] This transition moment couples with the transition moments of the DNA bases, resulting in a positive red-shifted CD, as mentioned earlier. How a second Hoechst 33258 molecule may bind to sequences with high AT content has been extensively discussed.[30,31,47] Bazhulina et al.[30] suggest that binding of a second Hoechst 33258 molecule creates a dimer in the form of a sandwich for $[poly(dAdT)]_2$, but with the monomer units allowed to slide relative to each other so that in poly(dA)·poly(dT) the dimer may cover an additional two base pairs. By way of contrast, Harris et al.[47] instead propose that only a single Hoechst 33258 molecule binds to the AT tract of A_4T_4. We shall return to these questions in the Discussion section. In principle, a second Hoechst 33258 could either bind as a true sandwich dimer or be accommodated in the minor groove just slightly overlapping the first one in a consecutive manner, or it could also sit in the minor groove with a small or large distance from the first ligand. Finally, it could bind externally to the negatively charged phosphates of the DNA backbone. Here, inspection of the induced CD can help us to distinguish which of these putative binding sites the second Hoechst 33258 molecule may prefer. If the second Hoechst 33258 molecule would form a sandwich on top of the first one (Figure 3, model A), their long axis transition moments would not be separated relative to their direction of propagation, and this would result in a positive CD, in agreement with what we do observe. However, the very close distance between the two dyes would create a strong degenerate exciton coupling, and for a sandwich dimer this would result in a strong blue shift dominating over the red shift seen for the nondegenerate coupling between the first Hoechst 33258 molecule and the DNA bases (338 to 353 nm). Since this strong blue shift is not observed, we may already at this point dismiss the formation of a sandwich dimer. Furthermore, under our conditions we cannot see any increment of the induced CD when the AT tract consists of only four or six AT base pairs (A_2T_2 and A_3T_3). It is when the

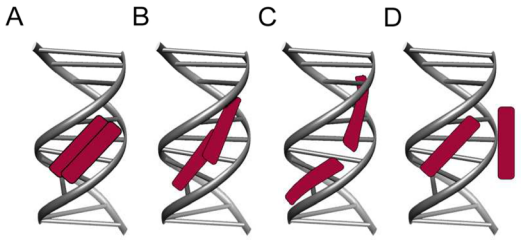

Figure 3. Schematic binding site models for two Hoechst 33258 molecules in the minor groove of dodecamer with central A_4T_4 target. The second drug molecule could either bind into a sandwich structure, on top of the first molecule (model A), or parallel in the minor groove and overlapping the first molecule (model B), or in the minor groove but with a slight distance from the first Hoechst 33258 (model C). Another potential mode of binding is external to the negatively charged backbone (model D).

AT tract is expanded to eight base pairs (A_4T_4) that we first detect this behavior, another strong reason to dismiss the option of the two dyes being sandwiched on top of each other. We can also dismiss the second model (contiguous) where the second Hoechst 33258 molecule binds in the minor groove, partly overlapping the first one (Figure 3B). Here again, the close distance between the dyes, and the chiral internal arrangement that the confinement in the groove will impose, would be expected to result in a conspicuous bisignate exciton CD pattern with approximately equal negative and positive CD signals, similar to the pattern seen for the DNA bases. This is not observed. Instead, the observed small red shift and continuous increment of the induced CD signal for the second Hoechst 33258 molecule fits best to the third model (model C), where the two molecules bind after each other in the minor groove with a certain separation from each other. Further evidence supporting this model (Figure 3, model C) is the following: first, the strong positive induced CD, which arises when Hoechst 33258 binds in the minor groove, is observed when both the first and the second ligand bind, suggesting similar binding sites and similar coupling modes with the nucleobases.[46] Second, the small red shift between the two binding sites is consistent with the expectation of only a very weak coupling between the two, approximately parallel, molecules positioned one after the other in the groove. In this proposed binding geometry, only the in-phase coupling mode occurring at the higher wavelengths is allowed, resulting in a red shift dependent on the distance r between the transition moments as $8r^{-3}$.[48,49] The distance r can be estimated by comparing the red shift of the induced CD of Hoechst 33258 to the red shift observed for the first strong transition in the hydrogen-bonded dimer of benzoic acid (see Discussion).

The CD results indicate some complexity with increasing binding ratios: the absence of an isodichroic point in the CD spectra when normalized with respect to ligand concentration shows that there are more than two bound spectroscopic species of Hoechst 33258.[46,49] However, this deviation does not prove the existence of more than two bound species. A single Hoechst 33258 molecule can be anticipated to prefer to sit in the middle of the minor groove of an A_4T_4 tract,[39] but when a second molecule binds, it probably has to push the first one to the side of the AT tract to share space in the groove. When these two molecules are sitting in proximity to each other in the groove, the original induced CD signal at 353 nm from the single Hoechst 33258 is no longer present. Instead a combined,

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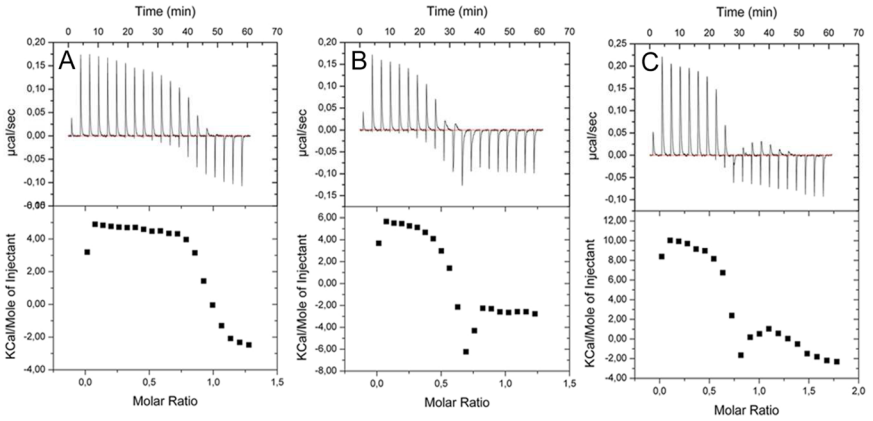

Figure 4. Raw ITC data for the titration of Hoechst 33258 into (A) A_2T_2, (B) A_3T_3, and (C) A_4T_4. Peaks at the top of each panel represent heat produced per injection, and at the bottom are shown integrated peak areas normalized per mole of DNA bases. Hoechst 33258 (210 μM) was titrated in 2 μL aliquots into the cell containing 206 μL of 39.8 μM A_2T_2, 40.5 μM A_3T_3, or 28 μM A_4T_4 solution at temperature 25.0 °C. A first small injection (0.5 μL) for each titration is discarded when data is analyzed.

and more red-shifted, induced CD from both molecules is detected.

It is noteworthy that the intensity of the induced CD signal from the second dye molecule does not increase with the same amplitude per added dye as the first one. This is an expected effect of weaker binding constant so that not all of the added Hoechst 33258, after reaching ratios above 1:1, binds to the second binding site in the A_4T_4 oligomer. Correspondingly, the absorbance spectra of Hoechst 33258 and A_4T_4 between ratio 1:1 and 2:1 are slightly shifted to shorter wavelengths compared to ratios less than 1:1 (Figure S2, Supporting Information), which is just an apparent effect of increasing concentration of free Hoechst 33258 contributing to the total absorbance spectrum. As expected, no such blue shift is seen in CD, which only monitors bound molecules. Above ratio 2:1, a slowly increasing shoulder at 330 nm in the CD spectrum is detected for A_4T_4, similar to the shoulders seen for A_2T_2 and A_3T_3 above 1:1. This shoulder most probably arises when Hoechst 33258 binds nonspecifically and externally to the negatively charged phosphate backbone of the DNA. Supporting this conclusion is the fact that we can see this behavior for all three oligomers after their AT sites in the minor groove are stoichiometrically filled. Also, the induced CD signal for this binding mode is weaker and at a wavelength very close to the absorbance maximum of free Hoechst 33258, which is in agreement with the Hoechst 33258 chromophore now being further away from the nucleobases.

Isothermal Titration Calorimetry. The raw ITC data of titration of Hoechst 33258 into the three oligomers is displayed in Figure 4. When Hoechst 33258 is titrated into A_2T_2 (Figure 4A), the curve demonstrates ΔH of +5 kcal/mol for binding at 25 °C in 200 mM NaCl, and the position and sigmoidal shape of the curve shows that the site is filled at 1:1, in agreement with the spectroscopic results. The endothermic character indicates an entropically driven process. However, when the titration was continued further, a constant value of −2 kcal/mol injectant for each titration was obtained. (The ITC measurements were continued up to ratio 4:1; data not shown.) The heat of dilution for Hoechst 33258, when the dye is titrated into pure buffer, is slightly endothermic, suggesting that the constant −2 kcal/mol injectant seen at the last injections of the titrations is indeed originating from nonspecific binding to DNA, and this value agrees well with previously reported results for nonspecific binding taking place after the minor groove is saturated.[20,43] Also, both A_3T_3 and A_4T_4 display a constant −2 kcal/mol of injectant after their minor grooves are filled, confirming that the binding is not related to the sequence of the oligonucleotide. Thus, we can conclude that this exothermic reaction represents most probably nonspecific external and mainly electrostatic binding to the DNA phosphate backbone. The ITC titrations for A_3T_3 and A_4T_4 are not as easy to interpret before this nonspecific binding takes place. The ITC data begins as expected, with endothermic enthalpy for the first binding in the minor groove, +5.5 and +10 kcal/mol for A_3T_3 and A_4T_4, respectively, and thereafter the start of the sigmoidal curves occurs. Interestingly, when the ratio 0.75:1 is reached, an exothermic dip is detected in the ITC data, and after this interruption the signal returns to its normal sigmoidal shape. Our enthalpic values for the different ITC titrations are all in close range to previously reported data for A_2T_2,[30,43] A_3T_3[18], and $[poly(dAdT)]_2$,[30] although these reports do not note any exothermic dip for Hoechst 33258, which could be explained by the technique's improved sensitivity in more recent years. By contrast, Freyer et al.[44,45] have observed the exothermic behavior in their ITC data for netropsin binding to an AATT-containing hairpin DNA construct before a ratio of 1:1 is reached. They propose two binding conformations of netropsin to AATT, with and without a bridging water molecule between the drug and the DNA, that might give different enthalpies. Although this binding model is somewhat speculative, it is consistent with the results. We have applied a similar model to our analysis of the CD and ITC data (see below). In addition, several control experiments were made to make sure that the exothermic dip seen in ITC is not simply because the concentrations of oligomers were higher in the ITC experiments than in the CD experiments, thereby promoting the formation of other species (40.5 μM for A_2T_2 and A_3T_3 and 27.8 μM for A_4T_4 in ITC, compared to 5 μM in CD). At high dye concentrations and/or at high dye:DNA ratios, several variants of dye−DNA aggregates can form, and the dye could self-aggregate. To avoid such effects, we have used the lowest detectable dye concentrations possible that still give reproducible enthalpy results. Buurma and Haq[20] have created a useful software package for combining self-aggregation and DNA binding for Hoechst 33258, but we have instead chosen to minimize the presence of self-aggregation by using lower dye

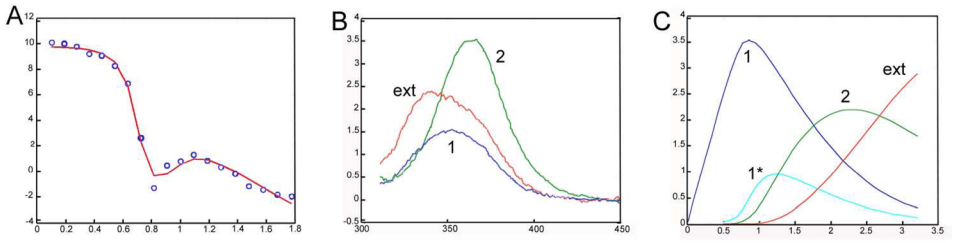

Figure 5. Illustration of best fits of measured signals to theoretical binding isotherm for A_4T_4: (A) CD data (red line) and ITC signal (blue circles). (B) Components of CD spectra according to SVD analysis: HD (blue), H_2D (green), and H_3D (red). (C) Concentration profiles, with the assumed identical HD (blue) and HD* (cyan) separated from each other for clarity.

concentrations during the ITC measurements. This is motivated here since our main data analysis is based on CD measurements at very low ligand concentration. As a control, we performed ITC measurements also at less than half the original concentration, and we repeated the CD titrations at the same concentrations as the original ITC experiments, and we still observed the exothermic peak (data not shown). We could not decrease the oligomer concentration in the ITC experiments to the lowest concentrations used in CD (5 μM duplex) as the resulting heats then became too small to give trustworthy signals. To exclude the exothermic dip being due to formation of aggregates or to a portion of the DNA duplex being present as a hairpin, we also carried out ITC titrations at 10, 15, 20, 25, and 30 °C, with very similar results (Figure S4A, Supporting Information). The exothermic dip becomes slightly more pronounced with increasing temperature, in agreement with previously reported ITC data for netropsin.[45] When the ITC peaks are examined more closely in the region of the exothermic dip, two separate and opposite enthalpic contributions that compete with each other can be distinguished (Figure S4B, Supporting Information): one fast exothermic contribution followed by a slower endothermic one. One has to bear in mind that parts of the slow enthalpic interaction might be missed when the integrated peak area is calculated, since a slow and weak signal could be mistaken for baseline drift. Finally, we verified that the exothermic dips for A_3T_3 and A_4T_4 are not a result of high salt concentration. The same pattern was seen when buffer containing 50 mM NaCl was used (data not shown).

Fitting of Theoretical Isotherms. All CD spectra for A_4T_4 (35), A_3T_3 (10), and A_2T_2 (8) were baseline-corrected, and the resulting matrix **M** for each titration was decomposed into the three matrices, **U**, **S**, and **V**, as described in the Experimental Section. The resulting diagonal matrix **S** consists of non-negative elements in decreasing order and significance. For both A_2T_2 and A_3T_3, only two components had significant singular values within 310−450 nm (the DNA signal was not taken into account), meaning that for these two oligonucleotides there are only two different binding modes of Hoechst 33258, most probably arising from binding to the minor groove and external binding. For A_4T_4, on the other hand, three **U** components had significant singular values, and thus this oligomer contains three binding sites for Hoechst 33258. The significant **U** and **V** columns for A_4T_4 multiplied by the square root of **S** are shown in Figure S3 (Supporting Information).

As noted earlier, following Freyer et al.,[44,45] we expanded the model isotherm for A_4T_4 to include two distinct thermodynamic populations of the oligomer duplex, denoted D and D*, with different affinity constants and reaction enthalpies for binding of the first Hoechst 33258 molecule but giving the same product HD. The equilibrium equations used for calculating the binding isotherms for A_4T_4 were $[H][D]K_1 = [HD]$, $[H][D^*]K_1^* = [HD]$, $[H][HD]K_2 = [H_2D]$, and $[H_2D][H]K_3 = [H_2DH]$, where K_1 and K_1^* are the affinity constants for Hoechst 33258 binding in the minor groove of DNA for the two thermodynamically distinct species duplex species, K_2 is the affinity constant for the binding of the second Hoechst 33258 in the minor groove, and K_3 is the affinity constant for the external binding. The equilibrium equations described above are the simplest equations found that produce good fits for the combined CD and ITC data (Figure 5A; Figure S3B, Supporting Information). For A_2T_2 and A_3T_3, only the CD data sets were fitted, using a simpler model with one minor-groove binding site and one external binding mode, $[H][D]K_1 = [HD]$ and $[H][HD]K_3 = [HDH]$, respectively. The obtained values for the affinity constants and ΔH are summarized in data set 1 in the Supporting Information.

Figure 5B shows the resolved CD spectra for HD, H_2D, and H_2DH for A_4T_4. The concentration profiles from the fitting (Figure 5C) indicate that the first ligand binds in the minor groove of D with high endothermic enthalpy, followed by binding to D* with exothermic enthalpy, and this explains the exothermic dip in the ITC data. The ratio of D to D* is 0.72:0.28. After the first binding site is filled, the second ligand fills in as H_2D with slightly endothermic enthalpy, and when this site is filled, external binding to the DNA backbone takes place, forming H_2DH.

^{1}H NMR Titration. Due to the lack of assignable base protons in the minor groove, binding of a ligand to this groove is monitored via the chemical shifts of imino protons and methyl protons of thymines. The latter are located in the major groove, and changes in their chemical shifts upon ligand binding in the minor groove have been explained by DNA conformational changes.[39,50,51]

In this study, the changes in chemical shift of these methyl protons are utilized as an indication of Hoechst 33258 binding to DNA. Addition of Hoechst 33258 to $(CGAAAATTTTCG)_2$, until equivalent numbers are reached, results in strong shifts and splitting of the central methyl peaks, T8 and T9, while the more peripheral ones, T7 and T10, are less shifted and symmetry is mostly preserved (Figure 6, dark orange). This indicates that Hoechst 33258 binds in the middle of the AT region. The peak splitting can be explained by breaking of the symmetry of the DNA spectrum as a result of the inherent asymmetry of Hoechst 33258. This binding of a first Hoechst 33258 molecule is in agreement with a previous NMR study.[39] Continued titration of Hoechst 33258 to DNA beyond 1:1 (dark orange in Figure 6A) results in only small spectral changes of the methyl peaks of T10 and T7. By contrast, at a ratio of 2:1 (blue), each of the split methyl peaks

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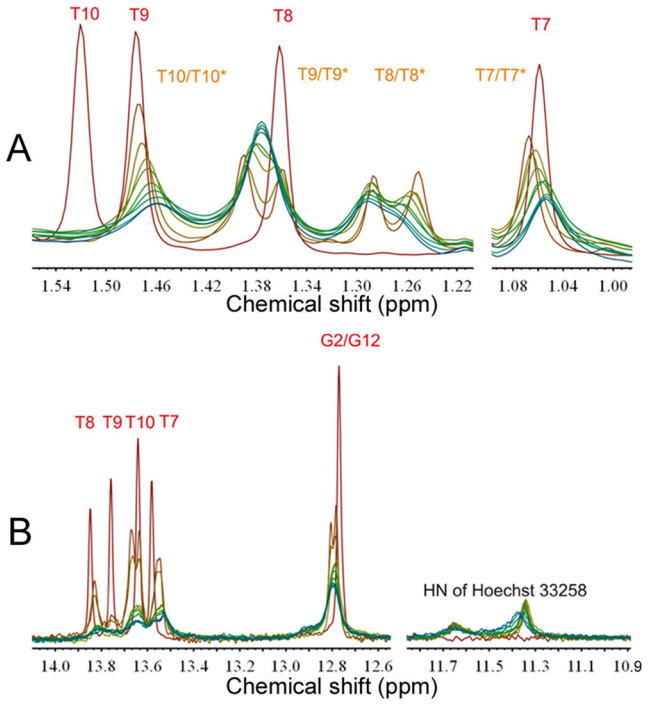

Figure 6. ^{1}H NMR spectra and assignments (800 MHz): (A) thymine methyl region (1.02−1.55 ppm); (B) DNA imino region (12.6−14.1 ppm) and region with NH protons of Hoechst molecule (11.1−11.8 ppm). Increasing Hoechst 33258 to DNA ratio is illustrated by color: 0:1 (dark red), 1:1 (dark orange), 1.1:1, 1.2:1, 1.3:1, 1.5:1, and 2:1 (blue).

of T8 and T9 is merged into one peak, hence resuming some of the lost DNA symmetry. With a second Hoechst 33258 molecule binding according to the sandwich model (model A), one would expect significant conformational changes of DNA, for example, widening of the minor groove, and consequently stronger chemical shift changes. The concomitant line broadening at higher Hoechst 33258 to DNA ratios indicates increased dynamic behavior, for example, caused by disruption of the optimal Hoechst 33258 to DNA binding interaction present at the 1:1 ratio. This behavior is consistent with two Hoechst 33258 molecules positioned one after the other in the minor groove (Figure 3C), implying some small relocation of the first Hoechst 33258 from its optimal central binding site to give place for the second ligand molecule; this relocation would not be necessary according to the sandwich model. The two HN resonances of Hoechst 33258 (11.7 and 11.3 ppm) are not split and move only marginally upon titration from 1:1 to 2:1, supporting a symmetrical arrangement of the two Hoechst 33258 molecules in the minor groove. By contrast, two Hoechst 33258 molecules near each other, as in models A and B, are expected to sizably influence each other's chemical shifts. The spectra recorded for ratios between 1:1 and 2:1 are closely described by sums of spectra for 1:1 and for 2:1, indicating the coexistence of both kinds of species.

DISCUSSION

The most important novel result of this study is resolving the controversy of binding mode for the second Hoechst 33258 molecule entering a ligand−DNA complex, where one Hoechst 33258 molecule is positioned in the minor groove of a short A_nT_n sequence. The binding mode of a second minor-groove binder has been the subject of debate for a long time, for Hoechst 33258 as well as for other analogous minor-groove binders.[1,3,11] The understanding of how multiple minor-groove binders are accommodated in DNA is relevant to address for a number of reasons: for example, in context of targeting nucleic acid sequence with sandwiched polyamides able to discriminate A and T in a sequence.[52] The literature contains early observations of binding dualism: that DNA-intercalating cyanine dyes may form groove-bound stacked aggregates,[40,41,53] and vice versa, that minor-groove binding drugs in absence of AT regions bind by intercalation.[40,41] In order to characterize both binding site size and binding mode for Hoechst 33258, we have chosen to study short A_nT_n oligonucleotides (n = 2, 3, and 4), for which the effects of binding to AT regions of different lengths can be easily tested.

The distinct qualitative difference between the CD spectrum when Hoechst 33258 is titrated into a solution of an oligonucleotide containing an A_nT_n stretch of eight base pairs (A_4T_4) and the spectrum when it is titrated into an oligonucleotide containing six base pairs (A_3T_3) suggests that two ligands can indeed enter the minor groove of A_4T_4, but only one ligand fits in the shorter AT region of A_3T_3. Also, the CD spectra for the oligonucleotide containing four AT base pairs (A_2T_2), behaves very much like A_3T_3, suggesting that the ligands bind to these sequences in a similar manner. It is confirmed that Hoechst 33258 covers approximately 4 base pairs in the minor groove of AT sequences, indicated by both models and crystal structure for the monomer complex;[37] thus only one Hoechst 33258 is expected to fit into A_2T_2 and A_3T_3, in agreement with our findings. When the binding mode of the second Hoechst 33258 to A_4T_4 is further analyzed, our spectroscopic and thermodynamic data support the conclusion that the two ligands are both sitting in the minor groove some distance apart, not in the form of a sandwich structure as previously proposed.[31] We will discuss the structure and nature of this binding, but let us first put our study into context.

The class of minor-groove binding molecules represented by Hoechst 33258, together with the considerably shorter 4′,6-diamidino-2-phenylindole (DAPI) and 2,5-bis(4-amidinophenyl)furan (APF), has been extensively studied with respect to binding to DNA. Flow linear dichroism (LD) spectroscopy has proven that all three dyes exhibit a binding angle of approximately 45° from their long axis relative the helix axis of AT-rich DNA, in agreement with an edgewise insertion of these dyes into the minor groove.[40,54−56] The near-UV absorption of Hoechst 33258 is due to two electronic transitions occurring close in energy and polarized close to the longest dimension of the molecule. An angle of 46° ± 2° for the average inclination relative to the DNA helix axis for these two close-lying transitions at 315 nm (19° to dye long axis) and 280 nm (23° to dye long axis) have been determined by LD spectroscopy.[16] This solution binding geometry is in excellent agreement with recent crystal data that show a planar conformation edgewise inserted in the minor groove at 45° and covering 4.5 base pairs.[37] By way of contrast, in a pure GC context, LD shows an angle of approximately 90° (±15°) between dye long axis and helix axis, thus excluding minor-groove binding and possibly suggesting intercalation.[40] The minor-groove binding, however, is restored in [poly(dI-dC)]$_2$ as a result of removal of the exocyclic amino group of guanine.[57]

Our CD data, which give a single strong induced CD at the position of the near long-axis polarized transitions in Hoechst

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33258, are consistent with nondegenerate exciton theory,[46,58] crystal structures,[22,32−37] and LD measurements for the binding of one Hoechst 33258 to an AT region,[16] confirming that the ligand indeed binds in the minor groove for all the oligomers. The binding of a second Hoechst 33258 molecule, as partly discussed in the Results section, will be further elaborated on here. Figure 3 illustrates four schematically different structures of alternative binding modes for two Hoechst 33258 molecules to A_4T_4. One possibility is that the second Hoechst 33258 binds to the complex as a sandwich dimer (Figure 3A), which would be consistent with the polyamide hairpin structures studied by Dervan et al.[8,59,60] However, in such a case, degenerate exciton coupling between the two almost parallel chromophores would have given rise to a very strong interaction: if perfectly parallel, only the in-phase high-energy combination would survive, leading to a strong blue shift, contrasting the observed spectral behavior. Such a geometry can be excluded also from the fact that the interaction is obviously weaker than the interaction with DNA bases, which is responsible for a significant red shift and hypochromicity compared to the free Hoechst 33258 chromophore. The sandwich hypothesis (model A) can also be dismissed on the basis of our NMR results: sandwich stacking would introduce big chemical shifts for the imino protons of Hoechst 33258, in disagreement with the very small changes observed (Figure 6B). We can therefore, from all spectroscopic evidence, rule out the sandwich alternative. We independently reached this conclusion also from the spatial arguments and observation that a second Hoechst 33258 molecule is not allowed to enter into the A_3T_3 context.

Another possibility, that the second Hoechst 33258 molecule is sitting parallel with the first one but with a certain overlap (contiguous), is shown by model B in Figure 3. This binding geometry is unlikely, though, since if such an overlap were allowed, it would have been detected for A_3T_3 as well, which is not the case. An additional piece of evidence disfavoring a contiguous structure is that in such a case a preferred chiral arrangement of the monomer units relative to each other would be expected, due to the helical rise of the groove, which should be accompanied by a strong exciton CD with two bands of equal strength but opposite CD, one at the high-energy side and one at the low-energy side of the monomer absorption. No such extra CD is observed, and the size and sign of the induced CD for both the first- and second-binding Hoechst 33258 chromophores are in agreement with what is expected solely by interaction with DNA base transition moments.

The third possibility is that the two Hoechst 33258 molecules are bound separately, one after the other (Figure 3C). Being approximately parallel, only the in-phase long-axis polarized coupling mode is allowed, in this case expected to contribute a red shift to the absorption. The three possible molecular orientations, head−tail, head−head, and tail−tail, should all give the same excitonic behavior (red shift) but are from an electrostatic point of view different. If "head" represents the positively charged methylpiperazine end, the repulsive head−head arrangement should not be very likely. The magnitude of the red shift should be proportional to the dipole strength (square of transition moment) of the absorption and inversely proportional to the cube of the internuclear separation. The situation is analogous to coupling of the charge-transfer transitions of two benzoic acid chromophores connected by ring carboxylic acid hydrogen bonds to a linear dimer, and we may use the distance as a scaling parameter to get a rough estimate of how far away from each other the units in the dimer could be. The energy shift between the (allowed) in-phase and the (forbidden) out of-phase exciton absorption peaks for the parallel arrangement is

$$2V = -4K\mu^2 R^{-3}$$

$$\Delta\nu_{m-d} = K'\varepsilon_{max}\Delta\nu_{1/2}R^{-3}$$

where μ^2 (square of transition moment) is equal to the dipole strength, R is the separation between the transition moments' centers, and K is a constant. Utilizing the second (scaling) form of the equation, where K' is a new constant, ε_{max} is the extinction coefficient at absorption maximum, and $\Delta\nu_{1/2}$ is the half-width of the absorption band, the frequency shift $\Delta\nu_{m-d}$ (both in frequency units) between the position of the monomer and dimer absorption peaks suggests that R is 13−18 Å. Since the length of the Hoechst 33258 molecule estimated from the crystal structure is about 18 Å, this would mean that the two molecules are indeed nearly touching each other or might even be partly overlapping (contiguous geometry). However, this estimate of R should be taken for what it is worth, as the evaluation of the red shift and positions of the transition moments are subject to considerable uncertainty. The saturated piperazine ring is not part of the chromophore system, so the center of the transition dipole is on the middle aromatic system rather than in the middle of the molecule. In a more exact treatment, also the (nondegenerate) coupling with the DNA base transition moments, which are responsible for most of the red shift and here are treated only as a fixed offset, should be taken explicitly into account.

By combining the CD and ITC data, we can get a more detailed understanding of the binding modes. The first ligand that binds to the three oligomers is structurally the same as judged from CD, but by ITC we can see that the binding demonstrates two different enthalpic contributions for the first Hoechst 33258 molecule upon binding to A_3T_3 and A_4T_4, since we see an exothermic peak at binding ratio 0.75:1. Small variations in binding mode can be detected by ITC, allowing determination of binding free energy, enthalpy, and entropy. The technique is very sensitive and can detect differences in binding modes that other spectroscopic techniques fail to identify. In the global analysis of the combined ITC and CD data, the best fit is obtained when 72% of the duplexes are assumed to be in a high-affinity form and 28% in a low-affinity form. The most evident difference between A_2T_2, where no exothermic dip is detected, and A_3T_3 and A_4T_4, where the exothermic dip is present, is the length of the AT region. One could argue that the ligand can bind only in one specific place in A_2T_2, while for the oligomers with longer AT tracts the first ligand could bind in two different places, thus giving rise to two binding modes with different enthalpies, one endothermic and one exothermic. However, this could not be the case since it would not give rise to such a clear exothermic dip; it would instead be a combination of the two binding modes in each titration. We can exclude that bridging by dye molecules between two DNA molecules, or other aggregate or complex formation, is the source of the discrepancy, since such effects would have been detected in the CD or NMR titrations. It has been speculated whether the ligand may be presented to two different populations of oligomer, with slightly different minor grooves, for example, with different hydration patterns. Freyer et al.[44,45] observe a very similar exothermic dip in their ITC measurements for netropsin binding to AATT hairpin

constructs. They explain this behavior by the presence of two hydration states of the DNA, giving rise to two different binding enthalpies, which is consistent with our results that the two populations should give rise to identical products HD as judged by analysis of the CD titration. However, we can rule out this hypothesis by NMR titration. The pure DNA peaks of A_4T_4 decrease linearly in the NMR spectrum when Hoechst 33258 is added; thus there is no evidence for two different DNA populations (data not shown).

There is finally a possibility that the differences in time scales between ITC and the spectroscopic techniques can be important, in case ITC does not fully represent an equilibrium system. We observe in CD and NMR the structure of the fully equilibrated dye−DNA complex, while the ITC signal is an integrated time derivative, averaging fast and slow interactions and their associated heat evolutions. Thus, the accurate integration of slower equilibration processes of, for example, initially clustered ligands is difficult due to thermal noise and baseline drift.

Our NMR titration confirms the well-defined central minor-groove binding for the 1:1 complex and also supports the conclusion from the CD analysis: in order to accommodate two bound Hoechst molecules, the first one has to slide away from its preferred central site to give rise to a relatively symmetrical arrangement.

CONCLUSIONS

We demonstrate that when the AT-specific minor-groove binder Hoechst 33258 is added to a dodecamer containing an AT region of varying length (A_2T_2, A_3T_3, or A_4T_4), it binds with high affinity in the middle of the AT sequence. When the ratio of Hoechst 33258 to oligonucleotide is increased, it is only in the minor groove of A_4T_4 that the second ligand is binding. We conclude that when the second ligand molecule enters the minor groove of A_4T_4, the first one moves away slightly to give space, and that these two molecules are now both sitting symmetrically in the groove with a small distance apart, as schematically illustrated by model 3 in Figure 3. This is the only model of the ones proposed that is consistent with our experimental data.

ASSOCIATED CONTENT

Supporting Information

Additional text and four figures describing fitting of theoretical isotherms; CD spectra of Hoechst 33258 bound to $A_2T_2$and A_4T_4; U and V vectors from the SVD analysis; ITC raw data of Hoechst 33258 in A_4T_4; and calculated ΔH and K values for A_2T_2, A_3T_3, and A_4T_4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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