# Methylene Blue Intercalates with Triplex Poly(dT)*Poly(dA)·Poly(dT) but not Duplex Poly(dA)·Poly(dT)

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Methylene blue intercalates with triplex poly(dT*dA·dT) even though it binds to the precursor duplex poly(dA·dT) in the major groove; the switch may be related to conformational modifications of the polynucleotide structure upon binding of the third strand.

Sequence specific recognition of duplex DNA can be achieved through binding of a third strand in the major groove to form a triple helical structure; *e.g.* oligo-homopyrimidines can bind parallel to homopurine duplex sequences *via* Hoogsteen base pairing [Fig. 1(*b*)].[1] However, the therapeutic potential of 'antigene' oligonucleotides[2] is restricted by the instability of DNA triplex structures under physiological conditions. Three strategies are used to enhance the ability of oligos to form stable triple helices: backbone and base modifications as well as covalent attachment of intercalators, *e.g.* acridines, ellipticines, *etc.*[1a] In order to understand why intercalators exert such a stabilising effect, several studies have thus focused on the modes of binding of different ligands to triplex: while intercalators stabilise triplex,[1a,3–5] minor groove binders such as netropsin[4,6a] and distamycin[6b,c] do not, and methyl green, which is believed to bind in the major groove of duplex DNA, has no interaction with the triplex[7] since its only possible binding site is blocked by the third strand.

We report here that methylene blue [MB, Fig. 1(*a*)] a DNA probe and photocleavage agent,[8] intercalates in triplex poly(dT*dA·dT).† Although methylene blue has all the features of a 'classical' intercalator, this is nonetheless a surprising result since our recent studies on its sequence-dependent binding demonstrated an unusual non-intercalative interaction with poly(dA·dT).[9]

Fig. 2 shows absorption and LD spectra‡ for methylene blue with poly(dA·dT) and poly(dT*dA·dT).§ When bound to poly(dA·dT) the LD in the dye band was negligible compared to that in the DNA band, as previously reported.[9] This indicates that the dye is either approximately unoriented or has an average orientation with respect to the helix axis of 55°.[10] By contrast, with poly(dT*dA·dT) the LD in the visible band, which arises from a single long axis-polarised transition, is strongly negative. The $LD^r$ for the bound dye ($\lambda_{max}$ = 680 nm, $LD^r$ = −0.149) was of the same magnitude as that of the nucleobases ($\lambda_{max}$ = 260 nm, $LD^r$ = −0.157) which places the dye long axis approximately perpendicular to the helix axis, a strong indication that it is intercalated.

The induced CD spectra‡ of methylene blue with the two polynucleotides (Fig. 3) have different signs, indicating quite different binding geometries. Upon melting of the triplex to duplex when methylene blue is bound, a positive CD spectrum reappears, indicating that the dye reverts to its non-intercalative site when the third strand dissociates.

Intercalation with triplex was also supported by fluorescence excitation measurements since there was an indication of energy transfer from the bases to the dye[11] when bound to the triplex but not duplex (data not shown).

It was not clear from these spectroscopic studies whether methylene blue favoured binding to the triplex or the duplex.

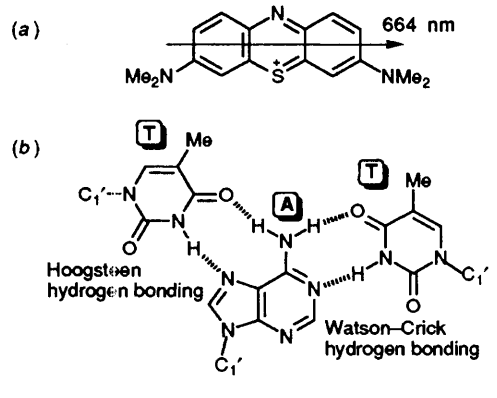


Fig. 1 The chemical structures of (*a*) methylene blue and (*b*) the (T*A·T) triplex

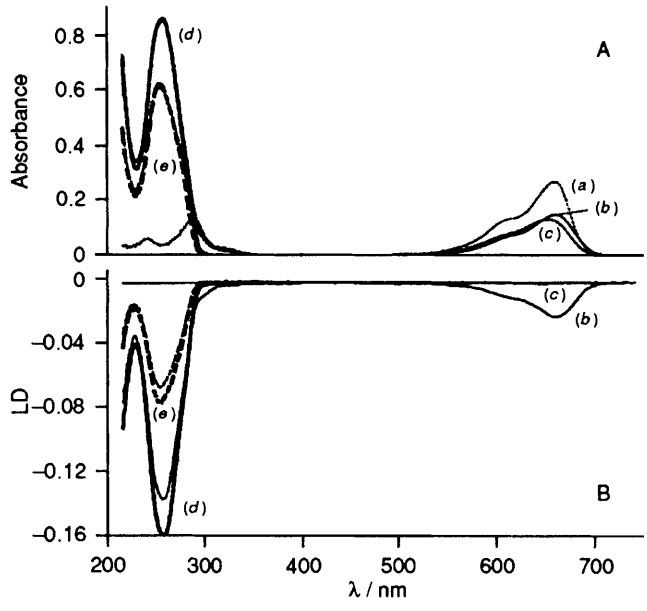


Fig. 2 Absorption and LD spectra for (*a*) free methylene blue (···) and (*b*) methylene blue bound to triplex poly(dT*dA·dT) (—) and (*c*) bound to duplex poly(dA·dT) (---) at P/D 50 (P = 150 μmol $dm^{-3}$). Spectra of the triplex and duplex in the absence of dye are shown as bold lines (*d*) and bold dashes (*e*), respectively.

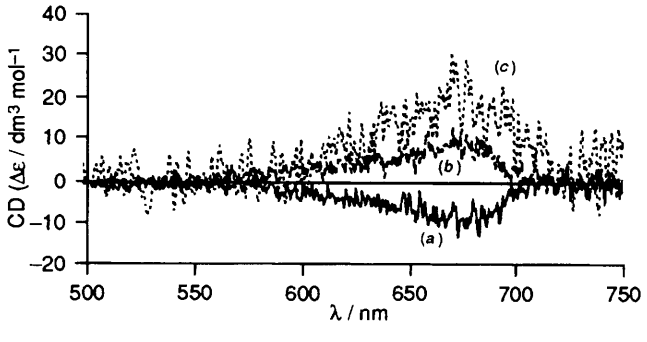


Fig. 3 CD spectra in the visible absorption region for methylene blue bound to (*a*) triplex poly(dT*dA·dT) (—) and (*b*) duplex poly(dA·dT) (—) at 20 °C. Also, (*c*) MB/poly(dT*dA·dT) at 55 °C after the first (triplex → duplex) melting transition (···): no CD is observed in the visible region after the second melting transition. P/D 50 (P = 150 μmol $dm^{-3}$).

CD melting studies at two P/D (nucleotide/dye) ratios demonstrated slight stabilisation of both triplex and duplex as shown by the small increases in their melting temperatures (Table 1). However, the stabilisation of triplex afforded by binding of methylene blue was small compared with other intercalating dyes,[3–5] indicating that intercalation with the triplex is not greatly favoured over major groove binding with non-alternating duplex.

We believe that major groove binding of methylene blue with the duplex is stabilised by a hydrogen bond between the dye ring-nitrogen and the adenine amino group in the major groove, although it is not clear why this binding mode is favoured over intercalation.[9] It has been reported that poly(dA·dT) has an unusual structure[12] with bifurcated hydrogen bonds between adjacent base pairs and large propellor twisting which could perhaps prevent intercalation (and certainly ethidium bromide intercalation is disfavoured[5]), but non-intercalative binding is also observed with alternating $[poly(dA\text{-}dT)]_2$ at high ionic strength. Additionally, the duplex binding mode appears to be quite stable with a reported binding constant ($10^5$ dm$^3$ mol$^{-1}$)[13] of the same order of magnitude as for intercalation in DNA:[14] thus, it is possible that the dye may bind in the groove because it presents a favourable environment and not simply because intercalation is hindered. Two important differences between the duplex and triplex are that in the triplex; (*i*) the major groove is filled by the third strand, the adenine $NH_2$ lone-pair acceptor site in the major groove is occupied, and there are no other lone-pair acceptor sites in any of the new grooves and (*ii*) the base-triplet may be more planar with less propellor twisting than the duplex base pair. Both these factors may play a role in the switch of binding mode, the first by excluding a favoured binding site and the second by making intercalation a more accessible and thermodynamically stable mode. However, the increased stability of the triplex on binding of methylene blue suggests that the dye has a preference for binding to the triplex and, therefore, conformational modifications in the polynucleotide structure on binding of the third strand may be the crucial factor influencing the change of binding mode.

These studies demonstrate the complexity and interplay of factors that determine binding mode; the interaction of a ligand with DNA depends not only its structure but also on the sequence and structure of the target.[15] The discovered novel binding behaviour of methylene blue provides deeper understanding of drug–triplex interactions and may aid in the improved design of 'anti-gene' drugs and triplex-specific ligands.

**Table 1** The effect of methylene blue binding on the thermal denaturation ($T$/°C) of duplex poly(dA·dT) and triplex poly(dT*dA·dT)

| | Triplex T*A·T[a] | | Duplex A·T[a] |
|---|---|---|---|
| Dye | $T_{m1}$ (ts → ds)[b] | $T_{m2}$ (ds → ss)[c] | $T_m$ (ds → ss)[c] |
| None | 33.4 | 64.8 | 63.3 |
| MB, P/D = 47[d] | 35.7 | 65.0 | 65.1 |
| MB, P/D = 23[e] | 37.4 | 65.7 | 66.4 |

[a] Polynucleotide concentrations were 150 μmol dm$^{-3}$ in nucleotides. Buffer was 20 mmol dm$^{-3}$ phosphate–2 mmol dm$^{-3}$ $Mg^{2+}$. [b] Determined from changes in CD at 208 and 218 nm. [c] Determined from changes in CD at 245 and 260 nm. [d] 23.5 base-pairs/dye and 15.7 base-triplets/dye. [e] 11.5 base-pairs/dye and 7.7 base-triplets/dye.

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## Footnotes

† (*) Represents Hoogsteen hydrogen bonding between bases and (·) represents Watson–Crick hydrogen bonding. Thus, poly(dA·dT) represents the anti-parallel duplex [poly(dA)]·[poly(dT)], and poly(dT*dA·dT) represents the triplex [poly(dT)]*[poly(dA)]·[poly(dT)] formed when the third strand binds in the duplex major groove parallel to the purine strand.

‡ LD spectra were measured on a JASCO J-500A spectropolarimeter adapted with an Oxley prism. Samples were flow-oriented with a shear gradient of 2500 s$^{-1}$. UV–VIS absorption spectra were recorded on a Varian Cary 2300 spectrophotometer. The reduced dichroism ($LD^r$) is defined as $LD^r(\lambda) = LD(\lambda)/A_{iso}(\lambda) = S(3/2)(3\cos^2\alpha - 1)$ where $A_{iso}$ is the absorption of the non-oriented sample. $\alpha$ is the angle between the absorbing transition moment and the DNA helix axis. For DNA, an effective angle of $\alpha = 86°$ is assumed.[10] CD spectra were measured on a Jasco J-720 instrument with a pathlength of 1 cm.

§ Measurements were conducted at room temp. on solutions buffered with 20 mmol dm$^{-3}$ phosphate–2 mmol dm$^{-3}$ $MgCl_2$. The triplex was prepared by mixing a 2 : 1 molar ratio of poly(dT) and poly(dA) in this buffer, incubating with stirring at 80 °C for 30 min and slowly cooling the solution to room temp.

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