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Determination of the stoichiometry of DNA–dye interaction and application to the study of a bis-cyanine dye–DNA complex

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Abstract

Some, though not all, cationic dyes exhibit enhanced fluorescence in the presence of DNA. Based on this phenomenon a simple and rapid method has been developed for determining the stoichiometry of interaction of such dyes with DNA. The method was validated using the well-characterised dyes Ethidium Bromide and Thiazole Orange dimer TOTO [1,1′-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methyl-idene]-quinolinium tetraiodide] as well as the butyl homologue of Thiazole Orange. In each case the stoichiometries thus determined were consistent with those already reported. Using this protocol the stoichiometry of interaction, with DNA, of a novel type of bis-cyanine dye was studied, and shown to involve binding of an unexpectedly large molar ratio of dye to DNA. Possible mechanisms of binding are suggested.

Keywords: Fluorescence enhancement; Cationic dye; Bis-cyanine; DNA

1. Introduction

The use of staining techniques for the detection and labelling of different types of cells and organelles is well known. Such methods have been widely applied to the identification and detection of naturally occurring macromolecules such as proteins and nucleic acids. For decades, radioisotopes had been the method of choice for the detection of DNA; however, increasing constraints on their use along with the demand for greater speed and sensitivity, has resulted in fluorescent methods dominating in many applications [1]. This shift towards utilising fluorescence in DNA detection is also due to a combination of recent developments including novel fluorescent probe molecules, better instrumentation, use of lasers to increase light input and improved methods of detecting and analysing fluorescent signals [2]. Fluorescence is now the most sensitive and readily available method for the study of many intermolecular interactions [3–5].

Recently, attention has been focused on the design of novel polymethine probes and stains. Polymethines are used extensively in biological, medical and drug development areas as fluorescent labels and probes [6] for cells, micelles and organelles [7–10], conformational studies via fluorescence energy transfer [11–13], flow cytometry [14–16], fluorescence microscopy [17], DNA sequencing [18,19], detection on microarrays [20], quantification of nucleic acids in capillary and gel electrophoresis [21–23] and single molecule detection of DNA [24]. The development of automated, high throughput screening (HTS) of drug candidates produced by combinatorial synthetic methods has been a significant new outlet for fluorescent probes and reagents [25,26].

Central to the characterisation of a dye–DNA complex is the establishment of its stoichiometry and mode of binding of dye to DNA, such binding interactions are known to proceed by several mechanisms: intercalation, groove binding and external stacking. As a first stage in the design of novel fluorescent dyes, as probes for DNA, a simple and reliable method was required for the determination of the stoichiometry of interaction of each speculative candidate dye with DNA.

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In order to ascertain this, increasing concentrations of dye were added to a given concentration of DNA. Initially, some dyes which interacted with DNA showed enhanced fluorescence, the magnitude of the enhancement increasing with the concentration of added dye. Thus when increasingly large aliquots of dye were added separately to constant concentrations of DNA the magnitude of fluorescence enhancement increased until DNA was ''saturated'' with dye, at which point further fluorescence enhancement ceased.

By this technique it was found that the known dye, Ethidium Bromide (**1**), combined with DNA in the molar ratio of 0.5 mol equiv dye per base pair of DNA. This is in accordance with published data. The *N*-butylated cyanine dye (**2**), a close relative of the known dye, Thiazole Orange (**3**), also interacted with DNA with similar stoichiometry: this was expected since it is known that the closely related methyl homologue, Thiazole Orange (**3**), is known to interact with DNA in the molar ratio of 0.5 mol equiv dye per base pair.

With the bis-cyanine dye TOTO (**4**), the ratio of dye:DNA base pairs was 1:4, again in good agreement with published data. Thus the technique appeared to offer a rapid and convenient method of determining the overall stoichiometry of interaction of the dye with DNA. Using this technique the binding of a novel bis-cyanine (**6**) was studied and an unexpected and unusually high stoichiometry with DNA was observed.

The above method thus provides a simple and rapid route to the determination of the stoichiometry of DNA–dye complexes. The optimum dye:DNA ratio, which is crucial for both linearity and sensitivity of DNA quantitation assays [27] is also revealed. Although high resolution structural studies, such as X-ray diffraction or NMR spectroscopy [28], are amongst the most powerful tools for providing a detailed insight into the mechanism of binding of the dye to DNA the establishment of the stoichiometry of interaction provides evidence to support or discount certain binding modes.

2. Materials and methods

TOTO (Fig. 3) was purchased from Molecular Probes as a 1 mM solution in DMSO, the butyl homologue [(**2**), Fig. 3] of Thiazole Orange and the bis-styryl cyanine dye [(**6**), Fig. 3] were from Avecia, and Ethidium Bromide and λ Hind III Digest DNA were purchased from Sigma. Stock solutions (20 mM) of Ethidium Bromide and the Thiazole Orange homologue (**2**) were prepared in DMSO. Working solutions of all the dyes and DNA were prepared by further dilutions in 0.1 × TE buffer pH 8. The purity of each non-commercially available dye was confirmed by elemental analysis and mass spectrometry. Elemental analyses of carbon, hydrogen and nitrogen were carried out on a Carlo Erba EA1108 elemental analyser at the Department of Chemistry, University of Manchester. Mass spectrometry was conducted by Hall Analytical Laboratories Ltd, Manchester using an MS MALDI-TOF LCT. UV–Visible absorption spectra were recorded on a Camspec UV–VIS spectrometer, slit width 10 nm, using quartz glass cells with pathlength 1 cm. Fluorescent emission spectra were recorded using a Tecan Safire microplate reader; excitation was set at sample absorption maximum and slit widths set at 10 nm, assays were carried out in 96-well, black microplate.

A 10 μM solution of the dye was prepared in TE buffer pH 8 and a further 10 two-fold serial dilutions were prepared. One hundred microlitres of each sample was pipetted into lanes 1–11 of rows A and B of the microplate, lane 12 received a blank of 100 μL of the buffer. For the binding isotherm, DNA stock solution (100 μL) was pipetted into each well in lane A. For the dye control profile, 100 μL of buffer was added to each well in lane B. In this way dye, DNA and buffer controls are assessed.

Subsequent dye samples were added to the plate using the above method (Fig. 1).

Plots of fluorescence enhancement (the difference between the level of fluorescence in the presence and absence of DNA) against the molar ratio of dye:DNA base pairs were recorded (Fig. 2). Thus, as increasing concentrations of dye were added

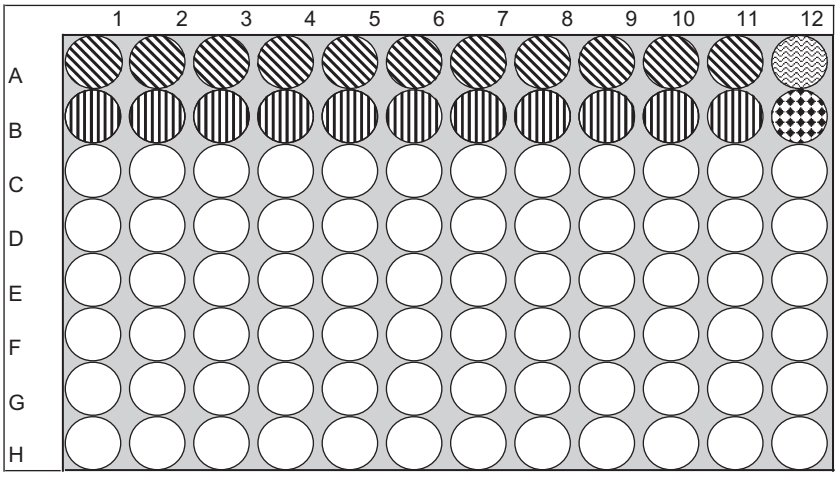

Fig. 1. Layout of microtitre plate A1–A11 serial dilutions of dye and buffer (diagonal lines), A12 buffer only, buffer control (zigzag lines), B1–B11 serial dilutions of dye and fixed concentration of DNA (vertical lines) and B12 DNA only, DNA control (diamond pattern).

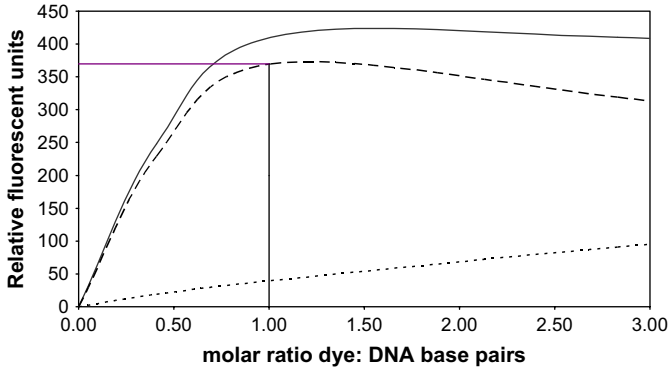

Fig. 2. Fluorescent emission of DNA–dye complex, dye and the calculated fluorescence enhancement (solid line = total fluorescence, short dash line = free dye fluorescence and long dashed line = fluorescence enhancement of bound dye).

stepwise to a fixed amount of DNA, fluorescence enhancement increased until the "saturation" level, of dye on DNA, was attained. Molecular modelling of dye structures was conducted using ab initio 6-311g** basis sets using Hyperchem 5.01 by Hypercube.

3. Results and discussion

The visible absorption and fluorescent emission spectra of the dyes are summarised in Table 1. Also listed are the stoichiometries of each dye per base pair.

The magnitude of the fluorescence enhancement was assessed for varying concentrations of each dye in the presence of a constant concentration of DNA. Thus, the magnitude of

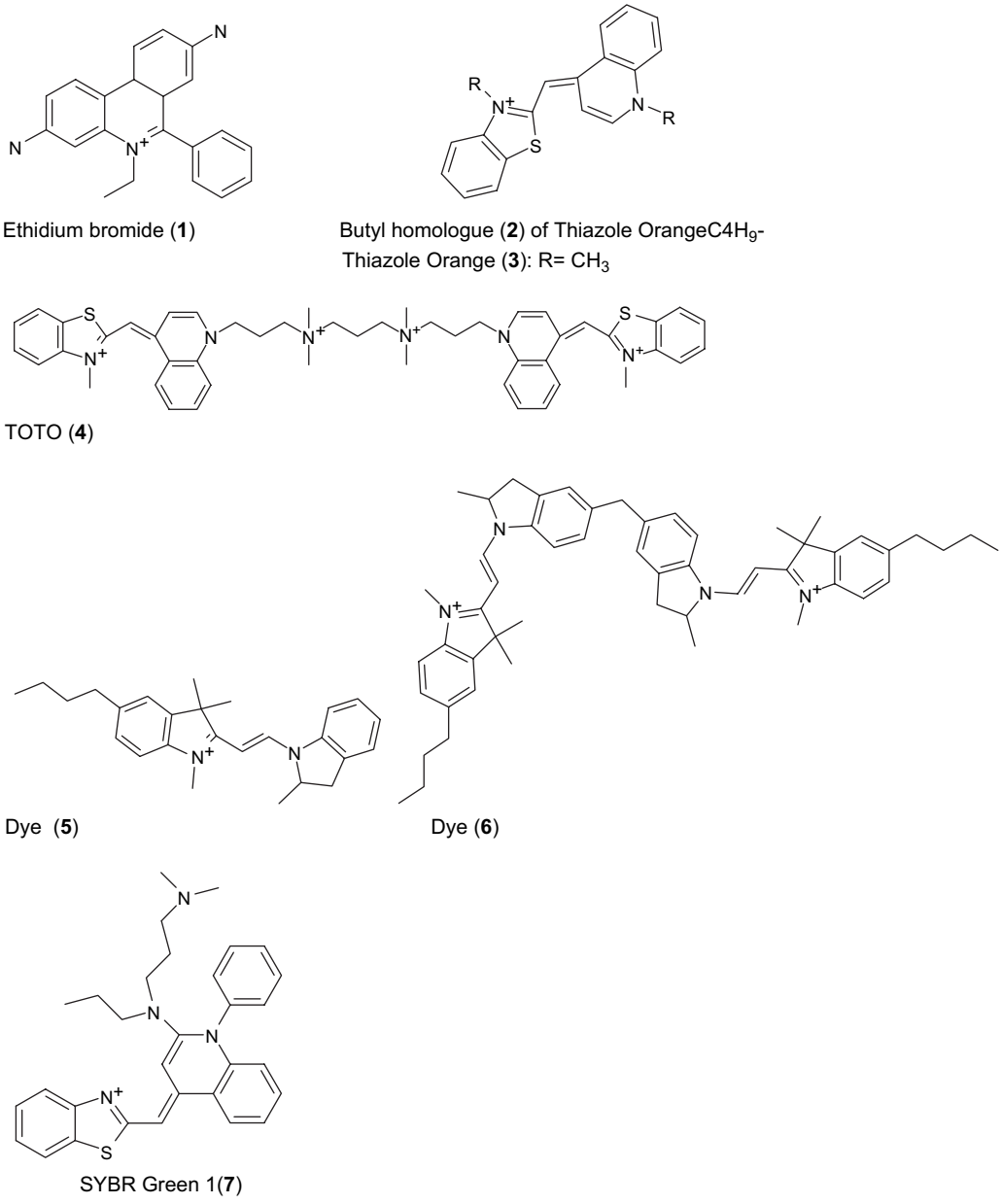

Fig. 3. Structures of the dyes investigated.

Table 1
Spectroscopic data of the dyes investigated

Name	e (mol^{-1} cm^{-1})	λ_{max} (nm)	F_{em} (nm)	F_{em} (DNA) (nm)	Dye:base pair ratio	ΔF
Ethidium Bromide	5700	595	620	610	0.5	36
Thiazole Orange	56,000	504	0	540	0.5	610
TOTO	117,000	514	0	534	0.25	782
Dye **5**	50,000	422	500	500	n/a	0
Dye **6**	99,000	435	470	584	2.88	670

the fluorescence enhancement of Ethidium Bromide in the presence of DNA is shown in Fig. 4, it can be seen that this enhancement increases progressively until it reaches a maximum, and thereafter it declines. This maximum represents the ''saturation'' binding level of dye to DNA which results in fluorescence enhancement. This maximum ratio is 0.5 equiv of Ethidium Bromide per base pair. This is in accordance with the accepted maximum value for intercalation based on the neighbour exclusion model. The binding of Ethidium Bromide has been shown to be characterised by two modes; at low dye:DNA ratios the major mode is intercalation, which is accompanied by fluorescence enhancement, while at high dye:DNA ratios the mechanism of binding is by non-specific external stacking, which does not lead to fluorescence enhancement [29].

The flourescence enhancement of the Thiazole Orange homologue (**2**), Fig. 5, demonstrates that the stoichiometry of the dye: DNA complex is similar to that of the Ethidium Bromide: DNA complex, 0.5 equivalents of dye per base pair. It has been shown that Thiazole Orange binds to double stranded DNA as a monomer at low binding ratios in an intercalative manner with an accompanying fluorescence enhancement, and that it binds to DNA at high binding ratios as a dimer which exhibits no fluorescence enhancement [30]. Owing to the structural similarity of the butyl homologue (**2**) to Thiazole Orange itself (**3**) it is reasonable to assume that both interact with DNA in a similar manner.

The fluorescence enhancement of TOTO (**4**), Fig. 6, shows saturation of the fluorescent complex at 0.25 mol dye per DNA base pairs. TOTO has been shown to bind by a mechanism of bis-intercalation [31], where each chromophore is intercalated between base pairs. Adherence to the neighbour exclusion principle would give saturation at 0.25 mol equiv dyes per base pair, in good agreement with the experimentally determined value.

A recent study [32] describing the structure and binding mode of SYBR Green 1 (**7**) to DNA reported that initially binding took place by intercalation which was not accompanied by the enhancement of fluorescence; at higher concentrations of added dye binding occurred at the surface and was accompanied by enhanced fluorescence. At pH 8, it would be expected that the pendant dimethyl amino group of SYBR Green 1 would be essentially protonated and therefore be expected to bind strongly, by ion–ion attraction to the anionic phosphate groups of DNA.

Fig. 7 depicts the increase in the enhancement of fluorescence as increasing amounts of the bis-cyanine dye (**6**) are added to a given concentration of DNA. Unlike the dyes described above the fluorescence enhancement does not initially increase linearly; fluorescence enhancement becomes greater at higher dye:DNA ratios until saturation is reached at 2.8 mol dye per mol of base pair. This stoichiometry of saturation of the fluorescent complex is far higher than that of the previous dyes examined.

The fluorescence enhancement of polymethine dyes bound to DNA is attributable to the fact that on photoexcitation a lack of free rotation around the internuclear bridge makes isomerisation around the C–C bonds of the polymethine chain impossible [33]; and subsequently nonradiative deactivation of the excited state is not possible causing the dye to fluoresce. Bis-cyanine dye (**6**) exhibits fluorescence enhancement up to the stoichiometry of 2.8 mol equiv dye per base pair, suggesting all dye molecules must be held in an increasingly rigid conformation until saturation.

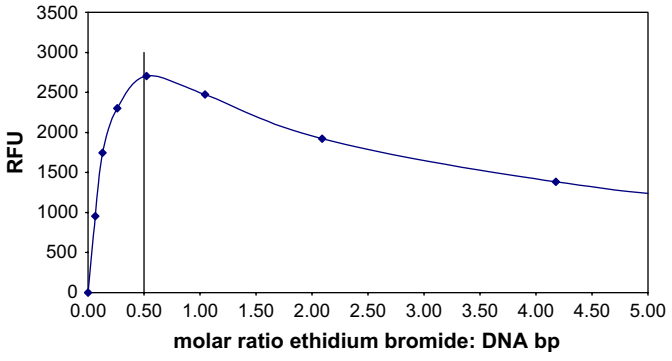

Fig. 4. Fluorescent emission at various DNA:dye molar ratios for Ethidium Bromide.

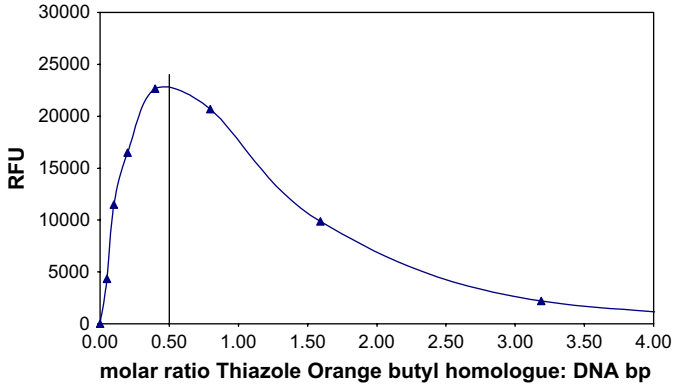

Fig. 5. Fluorescent emission at various DNA:dye molar ratios for Thiazole Orange butyl homologue.

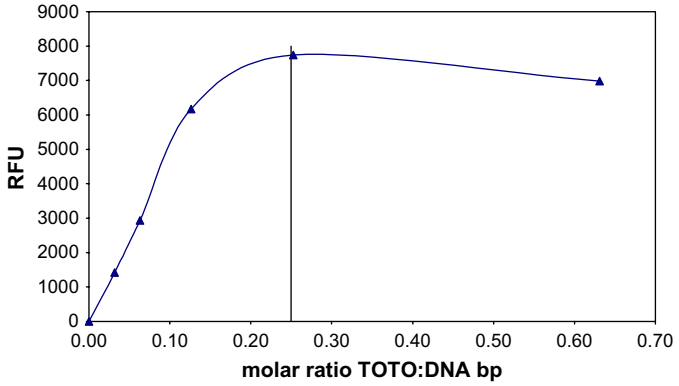

Fig. 6. Fluorescent emission at various DNA:dye molar ratios for TOTO.

Figs. 8 and 9 show the fluorescent emission spectra for the monomeric parent dye (**5**) and the bis-styryl analogue (**6**), respectively, the dimeric dye has marginally lower intrinsic fluorescence than the monomeric parent dye probably due to greater flexural rigidity of the homodimeric dye. The monomeric dye exhibits no enhancement of fluorescence when in the presence of DNA, consistent with the dye failing to form a complex with DNA.

Several explanations of the interactions of dyes with DNA have been put forward. Amongst the most well known of these are intercalation and groove binding.

In the intercalation model, dye is held between base pairs: the maximum concentration of dye that can be bound by this mechanism is thus 0.5 unit of dye per base pair. In groove binding the dye is held within the groove of the DNA where the close proximity of the dye to the walls of the groove allows for Van der Waal's forces and hydrogen bonding to stabilise the complex.

It is difficult to explain the uptake of 2.8 unit of the dimeric dye (**6**) per base pair using a groove binding model. The length of the minor groove of one complete turn of B-DNA helix is 40 Å based on the pitch of 34 Å and a diameter of 20 Å. Some molecules are known to form dimers in the minor groove of DNA [34]. Molecular modelling of the bis-cyanine dye (**6**) indicated the length of the dye to be 18 Å. This suggests that if an end-to-end aggregate structure is formed, the

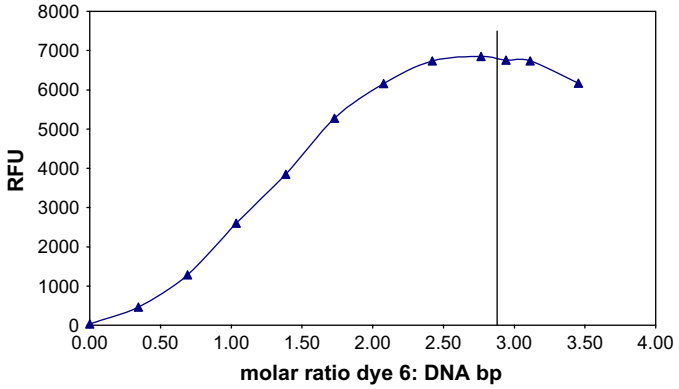

Fig. 7. Fluorescent emission at various DNA:dye molar ratios for dye **6**.

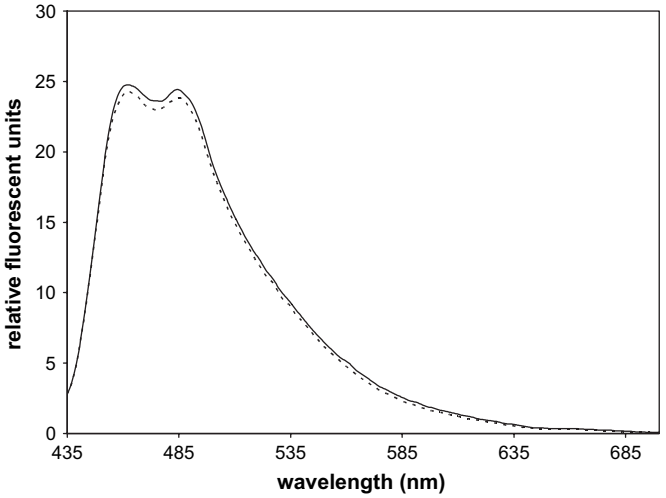

Fig. 8. Fluorescent emission of dye **5** (dashed line) in the presence of DNA (solid line).

minor groove could accommodate four dye molecules within one complete turn of the helix. This would give a stoichiometry of 0.4 mol equiv dye per base pair. Evidence to support this model has been previously reported [35]. Other styryl cyanine dyes have been demonstrated to bind in the minor groove of the DNA [36]. However, the large stoichiometry of 2.8 mol equiv dyes per DNA base pair prompts the question of how so many dye molecules can be accommodated in the DNA groove, or elsewhere, and in such a manner as to exhibit fluorescence enhancement.

It appears that none of the conventional modes of binding can readily accommodate such a large concentration of dimeric cyanine (**2**), 2.8 mol dye, or 5.6 equiv of monomeric chromophore unit, per base pair. Thus an alternative binding mechanism must be invoked to explain this observation.

The fluorescent emission of the dimeric dye exhibits a marked increase when in the presence of DNA, suggesting complex formation. These changes are very dependent on the ratio of dye to DNA. At increasing concentrations of dye, an intense new emission band appears at the much longer

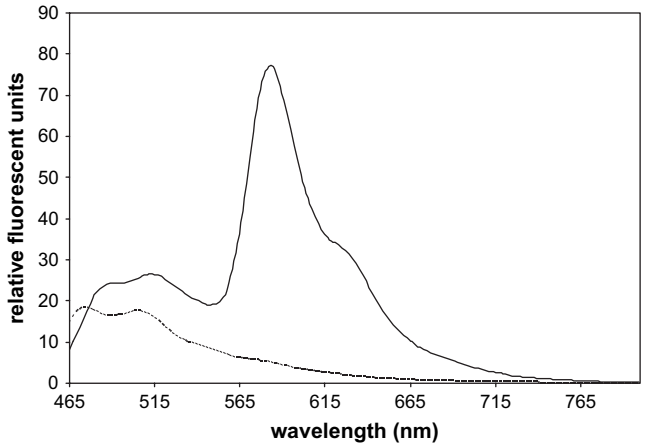

Fig. 9. Fluorescent emission of dye **6** (dashed line) in the presence of DNA (solid line).

wavelength of 600 nm, accompanied by a reduction in the 500 nm band. It is well known that cyanine dyes form aggregates in aqueous solution [37,38] and in the presence of DNA [39–42]. In aqueous solution, the bis-styryl cyanine dye (**6**) obeyed Beer's Law at concentrations up to 4×10^{-6} M, the concentration range over which interaction with DNA was studied, indicating, at least in true solution, that the dye exists only as a monomer and that no aggregation takes place in the absence of DNA. However, it has been demonstrated that cationic dyes are capable of forming aggregates on anionic surfaces and macromolecules more easily than in solution [40]. Bis-cyanines with an angular or parallel arrangement of the chromophores aggregate in weak polar solutions more easily than the corresponding monomeric cyanines [37]; the aggregates are built up from ion pairs with the type of aggregate structure depending upon the kind of substituents present, these aggregate structures have been characterised as H and J aggregates. The most important structural difference between the two types is the tilt angle; H aggregates have a structure in which the molecular planes stand perpendicular to the aggregate axis, whereas in J aggregates the molecular planes stand at an angle of 55° to the aggregate axis, see Fig. 10; bulky substituents tend to support the formation of J aggregates. Cyanines with short polymethine chains prefer to form J aggregates [37]. Therefore, if it does aggregate, the bis-styryl cyanine dye (**6**) with its bulky butyl chain and short polymethine chain might be expected to form J aggregates. Support for J-aggregate formation can also be found spectroscopically; it has been established that long wavelength emission was the result of J-aggregate formation [40]. The long Stokes shift suggests that the π-electron systems of the aggregates undergo substantial changes in the excited state compared to the monomer. This large Stokes shift increases the sensitivity of the dye for the detection of DNA owing to the reduction of background fluorescence at the DNA–dye complex emission peak.

However, this does not explain the shape of the curve of fluorescence enhancement against the concentration of added dimeric dye (**6**), depicted in Fig. 7. This indicates that initially the magnitude of this effect, per unit of added dye, is less than at higher dye concentrations, until, at ''saturation'', further enhancement of fluorescence ceases. It is unlikely, even at low concentrations of dye, to involve intercalation, since the monomeric dye can be assumed not to intercalate, as evidenced by the absence of fluorescence enhancement. An alternative possible mode of binding to DNA, which might explain the above observations, is possible. At lower concentrations of dye the primary attraction between dye and DNA is electrostatic: anionic phosphate groups on the external surface of DNA might be expected from ion–ion linkages with cationic

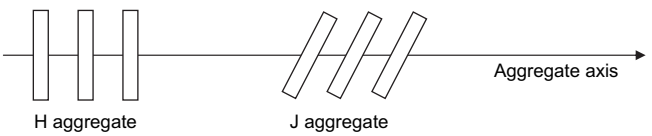

Fig. 10. Molecular structures of H and J aggregates.

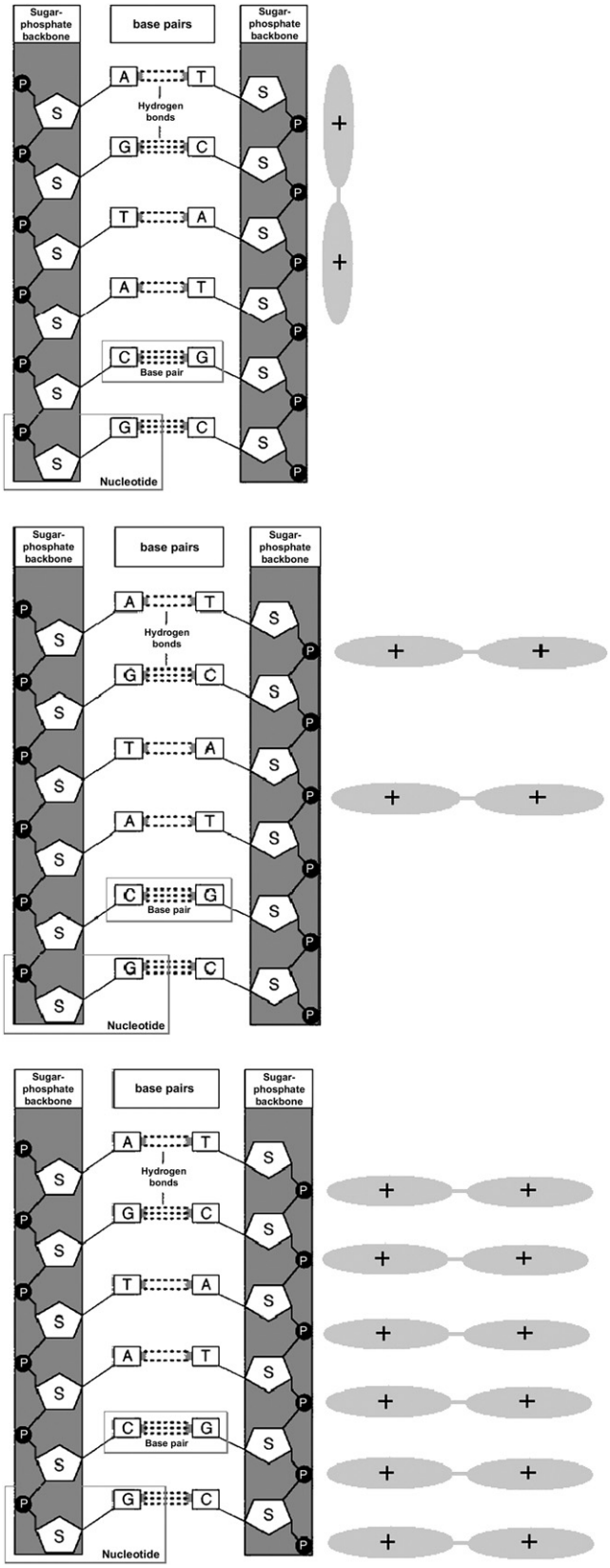

Fig. 11. (a) (top) Bis-cyanine dye (**6**) forming double ion–ion linkages and (b) (middle) simple ion–ion linkage. (c) Each bis-cyanine dye (**6**) forming one ion–ion linkage with every DNA phosphate backbone leading to cyanine dyes being held rigidly by ionic repulsion.

dye. Initially, at low ratios of dye to DNA this might be expected to be as depicted in Fig. 11; that is, each dye unit might form either of one, Fig. 11(a), or two, Fig. 11(b), ion–ion linkages with phosphate groups. These types of interactions might not be expected to inhibit movement around the cyanine axis and therefore would not be expected to lead to enhanced fluorescence. Alternatively, binding of the type depicted in Fig. 11(c) might restrict dye mobility resulting in fluorescence.

Interestingly, dye (**6**) showed a similar enhancement of fluorescence in the presence of poly(acrylic acid) (PAA) and poly(methacrylic acid) (PMA). In this environment the enhancement cannot be attributed to the intercalation or groove binding: most likely, it depends essentially on ion–ion attractive forces. Such attractive forces are also possible in dye–DNA binding; indeed such bonding in dye–DNA complexes might be expected to be marginally stronger than in dye–PMA complexes since the acidic monophosphate groups present in DNA are marginally stronger acids (pK_a, ca. 1) than the carboxylic acid groups present in the PMA, the pK_a of a typical aliphatic carboxylic acid is of the order of 4.75.

At higher concentrations of applied dye, as the anionic sites on the DNA are progressively occupied by dye, a point is reached where essentially all of the anionic phosphate units are involved in ion–ion linkages with bis-cationic dye. At this point the delocalised cationic nature of each cyanine unit that binds to the DNA is changed, thus positive charge accumulating to the vicinity of the phosphate (Fig. 12).

Additionally, as the free space around each phosphate unit is occupied by the dye, the second, unbound, cyanine unit of each dye will experience progressively greater electrostatic repulsion by neighbouring cationic cyanine groups: the effect of this will be effectively to fix the dye in a given conformation and thus inhibit the usual modes of energy dissipation, leading to enhanced fluorescence.

A mechanism of external stacking where one cationic dye molecule binds to an anionic phosphate group of a base would initially give a stoichiometry of 2 unit of dye for each base pair. Two further effects may explain the observed high stoichiometry. In unbound form, the cationic charge of a cyanine dye would be expected to be delocalised (see Fig. 12). If dye binds electrostatically to the anionic phosphate groups of DNA, thus forming a monomolecular sheath of dye around the DNA, this might effectively reduce charge delocalisation of the dye, producing a dipole capable of interacting with further cationic dye to form a bimolecular layer of dye on DNA. Additionally, due to the tilt angle of the structure of J aggregates a considerable portion of the π system is exposed which would allow for strong coupling to further dye molecules.

The observed lower fluorescence enhancement at lower stoichiometries would also be accounted for by this mechanism; the first few dye molecules binding externally to the phosphate groups on the DNA would not be sterically hindered and would be relatively free to exhibit photo-isomerisation leading to nonradiative deactivation of the excited state, however, as more dye molecules pack together and bind to the external phosphate groups of the DNA a more orderly structure would form resulting in restriction of movement and an inability of the dye to undergo photo-isomerisation leading the dye to fluoresce. In this arrangement the structure would gain

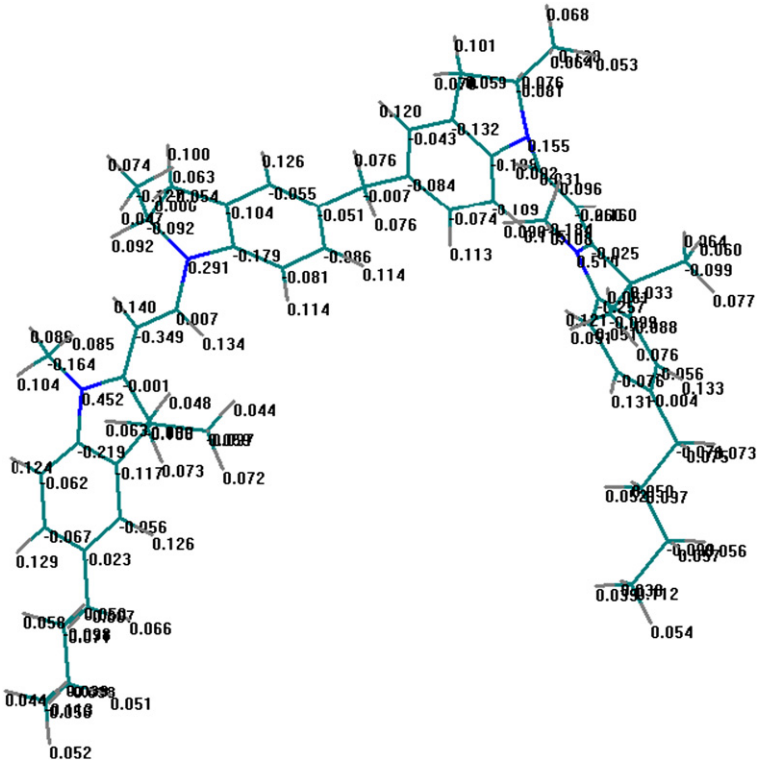

Fig. 12. Model of dye **6** showing partial charges.

stability through a variety of non-covalent interactions such as dipole–dipole and π–π interactions.

4. Conclusions

A rapid method has been established to determine the stoichiometry of binding of dyes with DNA and has been validated using the well-characterised dyes Ethidium Bromide, a Thiazole Orange derivative and TOTO.

A novel type of bis-styryl cyanine dye exhibited greatly enhanced fluorescence in the presence of DNA and was capable of binding to the DNA at very high concentration; interestingly the monomeric version of this dye exhibited no such fluorescence enhancement in the presence of DNA, and a novel mechanism is put forward to explain the observation.

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