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Supramolecular Self-Assembling Cyanine as an Alternative to Ethidium Bromide Displacement in DNA–Drug Model Interactions during High Throughput Screening

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Supramolecular self-assembling cyanine and spermine binding to genomic DNA was a model for DNA–drug interactions during high throughput screening. Spermine competitively inhibited the self-assembly of cyanine upon DNA scaffolds as signaled by decreased fluorescence from the DNA–cyanine J-aggregate. The sequence of DNA exposure to cyanine or spermine was critical in determining the magnitude of inhibition. Methanol potentiated spermine inhibition by >10-fold. The IC_{50} and association constant (K_a) in 16% methanol were 0.35 ± 0.03 μM and 2.86×10^6 M^{-1} respectively, relative to 3.97 ± 0.47 μM and 0.25×10^6 M^{-1} respectively, in buffer. Increasing concentrations of cyanine overcame spermine inhibition, demonstrating the reversibility of DNA–drug interactions. λDNA interacted similarly with spermine and cyanine, confirming system flexibility. The model drug, dye and methanol effects are discussed in detail. Cyanine might be a safer alternative to the mutagenic ethidium bromide for investigating DNA–drug interactions.

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Introduction

DNA–drug interactions interfere with transcription and replication,[1] and therefore critical to drug discovery.[2] Non-covalent DNA–drug interactions involve the intercalation of planar aromatic rings between DNA base pairs or binding of the drug to major or minor grooves in the DNA double helix.[3,4] Techniques to study DNA–drug interactions included gel shift, filter binding, nuclear magnetic resonance, mass spectrometry, calorimetry, surface plasmon resonance, dialysis, ultrafiltration, electrophoresis, ultracentrifugation, high-performance or thin-layer chromatography, and Raman, absorption or fluorescence spectroscopies.[2-4] Most of these technologies are time intensive or require sophisticated equipment and/or trained operators. Several are incompatible with high throughput screening (HTS) during drug discovery.[5]

One method to screen for DNA–drug interactions is "dye displacement," whereby dyes exhibit bright fluorescence upon binding to DNA that is followed by fluorescence loss upon dye displacement by the drug.[2,6] Dyes such as PicoGreen, SYBR Green I, cyanine[7] (and references therein), ethidium bromide (EB), Hoescht 33258 and 4,6-diamidino-2-phenylindole,[2] all bind to DNA accompanied by varying levels of fluorescence enhancement relative to the unbound fluorophore.[2] For example, EB fluorescence increases 24-fold upon binding to DNA and Hoescht 33258 undergoes 140-fold enhancement.[2] The most widely used dye in displacement reactions is EB,[2,6] despite its mutagenic, carcinogenic, teratogenic and toxic properties.[8] As an alternative to EB, we report a cyanine dye that spontaneously self-assembled upon DNA scaffolds accompanied by intense fluorescence from the J-aggregate.[7] We used DNA–spermine (polyamine) binding[9] as a model for DNA–drug interactions.[2-4] We describe various factors influencing the competition between cyanine and polyamine for binding to DNA and a 10-fold potentiating effect of methanol on binding. We carried out DNA–model drug interactions using 384-well microplates to demonstrate the capabilities for assay miniaturization, sample conservation, speed, robotics, liquid handling and HTS.[5]

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Experimental

Reagents and chemicals

Details of *Escherichia coli* genomic DNA (St. Louis, MO) and λDNA (Promega, Madison, WI) were published previously.[7] The polyamine spermine [$C_{10}H_{26}N_4$] (98% pure; moisture, 0.33%; M_r = 202.3) was from MP Biomedicals (Solon, OH). The FTIR spectrum of spermine conformed to standard. Spermine was dissolved in water as a 797-mM stock solution and stored at –20°C. Cyanine solution was prepared and used in the DNA binding assays as described previously.[7] Binding reactions were done in phosphate buffer (2 mM sodium phosphate, pH 7.5, 20 mM NaCl and 10 μM ethylenediamine tetraacetic acid), similar to a published buffer.[10]

DNA binding assay

The binding reaction consisted of DNA, cyanine and/or polyamine in a total volume (V_t) of 20 μL buffer in 384-well white microplates (Perkin Elmer, Waltham, MA). After 10 min incubation, the reactions were diluted to 100 μL using buffer or methanol–water (20:80, v/v), before taking fluorescence measurements (Molecular Devices M2 microplate reader,

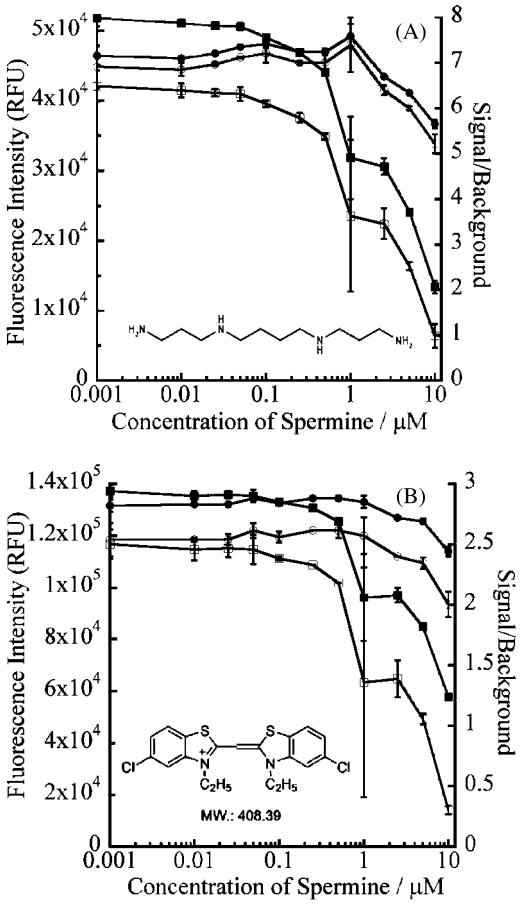

Fig. 1 (A) Spermine inhibition of cyanine binding to *Escherichia coli* genomic DNA with 425 nm excitation wavelength. Increasing concentrations of spermine were mixed with 1.2 fmol of DNA and 10 μM cyanine in 20 μL buffer. Fluorescence was measured at 470 nm. Open symbols represent fluorescence intensity and closed symbols *S*/*B*. Circles represent signal from reactions diluted using 20 μL of buffer and squares are reactions diluted with 20 μL of methanol–water. The inset shows the structure of spermine. (B) Spermine inhibition of cyanine binding to DNA with 450 nm excitation. The reactions from Fig. 1A were interrogated using 450 nm excitation/470 nm emission. The inset shows the structure of cyanine.

Sunnyvale, CA). Methanol toxicity was reduced by diluting to 20%. Volume change due to the addition of various reagents was <10%; therefore, no corrections were made.[11] Negative controls included buffer, DNA, cyanine, polyamine, and DNA + polyamine. Positive (100%) control was DNA + cyanine[7,11] and DNA + polyamine + cyanine represented competitive inhibition. Negative controls had fluorescence <10% of maximal fluorescence from the DNA-cyanine ensemble. Highest fluorescence from the negative control was used to correct the emission from the DNA-cyanine J-aggregate. Samples were excited at 425 nm and fluorescence was measured at 470 nm[7] except for Fig. 1B where 450 nm excitation was used. Emission intensity was expressed as relative fluorescence units (RFU).

Data analysis

Data were collected in triplicate and analyzed as described previously.[7] The concentration of polyamine inhibiting cyanine binding by one-half (50% drop in fluorescence from the DNA-cyanine J-aggregate) was designated as IC_{50}. The association constant (K_a) was calculated from the reciprocal of the IC_{50}.[11,12] The data were calculated as signal minus background ($S - B$) or as signal divided by background (S/B). To facilitate data comparison, the graphs also depict % changes to $S - B$ or S/B.

Results

Spermine inhibition of cyanine binding to DNA

Spermine produced a dose-dependent fluorescence decrease indicating a competitive interaction with cyanine for binding to *Escherichia coli* genomic DNA in buffer or methanol–water milieu. Results were similar when the reaction mixtures were examined at two different excitation wavelengths (Figs. 1A and 1B). The inhibition profiles were similar regardless of data analysis as $S - B$ or S/B. For example, preliminary IC_{50} values when analyzed as $S - B$ and S/B in methanol–water with 425 nm excitation were 3.0 and 4.0 μM, respectively (Fig. 1A). Likewise, the preliminary IC_{50} was 3.3 μM in methanol–water ($S - B$) at 450 nm excitation (Fig. 1B). Thus, reactions may be monitored for lower RFU and higher S/B by exciting the samples at 425 nm (Fig. 1A). Conversely, if higher RFU was desired, then 450 nm excitation may be used (Fig. 1B). These results were reproducible when the experiment was repeated 24 h later, demonstrating repeatability. In the absence of methanol, spermine produced a dose-dependent modest fluorescence decrease and a moderate drop in S/B; however, IC_{50} was not reached even at 10 μM spermine, regardless of the excitation wavelength (Figs. 1A and 1B). These data suggested a potentiating effect of methanol on the inhibitory effects of spermine upon cyanine binding to DNA. We scrupulously reported the results by including the single data point in methanol with a large error at 1.0 μM spermine out of a total of 11 on this particular tracing (Figs. 1A and 1B). The remaining 10 data points had small errors. Furthermore, it represented one data point with a large error amongst a total of 43 points profiled in Figs. 1A and 1B. Finally, we did not observe large errors in any of the other data. We therefore believe that our data are reproducible and reliable and we consider this single data point to be an outlier.

Methanol effects

We examined in detail the potentiating effects of methanol on the competitive inhibition of cyanine binding to DNA by spermine. The concentration of DNA, cyanine and spermine were fixed during the binding segment. Subsequent dilution of the reactions by adding increasing volumes of methanol–water led to progressively declining molar concentrations of all three reagents even though the amounts of the reagents were unchanged. For example, at 20 μL V_t in the absence of methanol, cyanine concentration was 10 μM and spermine concentrations ranged from 0.1 to 100 μM. When the reaction reached 100 μL V_t due to incremental additions of methanol–water, cyanine concentration decreased to 2 μM and spermine concentrations ranged from 0.02 to 20 μM due to 5-fold dilution. However, the ratio of spermine/cyanine did not change over the entire dilution range. We therefore profiled the data as fluorescence decrease (Fig. 2A) or decreases to %fluorescence (Fig. 2B) over increasing spermine/cyanine ratios. Volumes larger than 100 μL V_t were not attempted since maximal inhibition was between 16 and 16.7% (v/v) methanol (Fig. 2) and because the 384-microplate well capacity was reached.

Consistent with the data of Fig. 1, there was a methanol

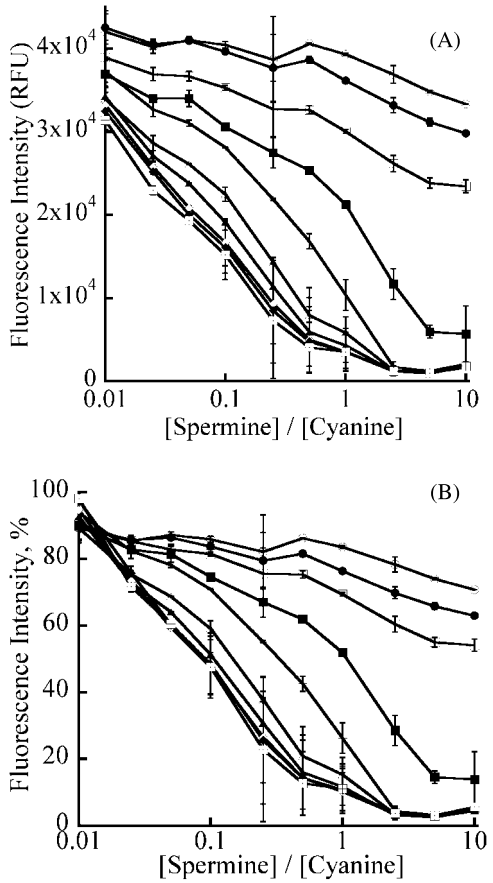

Fig. 2 (A) Effects of methanol on spermine inhibition of cyanine binding to genomic DNA detected by fluorescence intensity. Increasing concentrations of spermine were mixed with 1.3 fmol of DNA and 10 μM cyanine in 20 μL V_t. Reactions were diluted with increasing volumes of methanol–water and fluorescence was measured after each incremental addition of the solvent. Tracings represent fluorescence at different final concentrations (v/v) of methanol as follows: open circles, 0%; closed circles, 4%; open squares, 6.7%; closed squares, 10%; plus sign, 12%; open triangles, 13.3%; closed triangles, 14.3%; open diamonds, 15%; closed diamonds, 15.6%; open square enclosing plus sign, 16%. Cyanine concentration (μM) declined over this dilution range as follows: 10, 8, 6.7, 5, 4, 3.33, 2.85, 2.5, 2.22, and 2.0. (B) Effects of methanol on spermine inhibition of cyanine binding to genomic DNA detected by changes to percent fluorescence. All conditions are as described for Fig. 2A except that the fluorescence intensity was transformed into % values with emission in the absence of spermine being 100%.

dose-dependent increase in the diminishment of fluorescence, with maximal drop at ~16% methanol (Figs. 2A and 2B). At maximum dilution, fluorescence intensity in the absence of spermine decreased from 47085 ± 5586 RFU at 0% methanol (20 μL V_t) to 32170 ± 3677 RFU in 16% (v/v) methanol (100 μL V_t); a 31.7% drop in intensity. We therefore transformed the data as normalized percent changes to the fluorescence intensity (Fig. 2B). The inhibition profiles were similar regardless of the data analyses (compare Figs. 2A and 2B). For example, fluorescence decreased by one-half at spermine/cyanine ratio of 0.10 in 16% (v/v) methanol (Fig. 2A). Likewise, fluorescence declined by 50% at spermine/cyanine ratio of 0.09 (Fig. 2B). Furthermore, in both types of analyses, the IC_{50} values could not be calculated between 0 to 6.7% methanol, confirming the similarity of the analyses. To simplify data

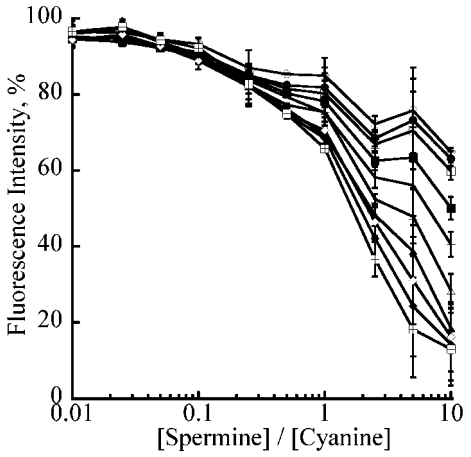

Fig. 3 Effects of buffer on spermine inhibition of cyanine binding to genomic DNA. All conditions are as described for Fig. 2A, except that incremental additions of buffer were used for dilution instead of methanol–water. Tracings represent progressively decreasing concentrations (μM) of cyanine identical to Fig. 2A as follows: open circles, 10; closed circles, 8; open squares, 6.7; closed squares, 5; plus sign, 4; open triangles, 3.33; closed triangles, 2.85; open diamonds, 2.5; closed diamonds, 2.22; open square enclosing plus sign, 2.0.

comparisons, subsequent results were profiled as changes to fluorescence, % or *S*/*B*, %.

Buffer effects

To exclude the possibility that the enhanced fluorescence diminishment was due to dilution and not due to methanol, we added equivalent volumes of buffer instead of methanol–water (Fig. 3). Identical, incremental additions of buffer were used as the incremental volumes of methanol–water in Fig. 2. Consequently, spermine/cyanine ratios varied identically in both experiments. Thus, except for the nature of diluent (methanol–water *versus* buffer), all reaction conditions were identical. It is clear that methanol enhanced the fluorescence decrease by >10-fold, since 50% fluorescence decrease was at a spermine/cyanine ratio of 1.3 in buffer (Fig. 3) relative to a spermine/cyanine ratio of 0.09 in 16% methanol (Fig. 2B). Similarly, at 10% methanol, IC_{50} was attained when spermine/cyanine ratio was 1.0 (Fig. 2B). By contrast, for the same dilution in buffer, IC_{50} was at spermine/cyanine ratio of 10.0 (Fig. 3). These data confirmed the potentiating effect of methanol upon spermine inhibition of cyanine binding to DNA.

Addition sequence

We tested the effects of exposing the DNA to spermine first, incubating for 10 min, followed by the addition of cyanine or reversing that order by exposing the DNA to cyanine first followed by the addition of spermine. Prior exposure of DNA to spermine decreased the J-aggregate fluorescence regardless of whether the reaction was diluted in buffer or methanol–water (Fig. 4). Consistent with the data of Figs. 1 – 3, fluorescence decrease was greater by diluting the reactions with methanol–water instead of buffer. Upon prior exposure of DNA to spermine followed by dilution using buffer, spermine enhanced the overall drop by 20% (50 to 30% residual fluorescence). On the other hand, dilution using methanol–water resulted in an enhanced 52% diminishment (60 to 8% residual fluorescence) (Fig. 4). This is remarkable since spermine/cyanine was 1.0 during buffer dilution, whereas in methanol–water, that

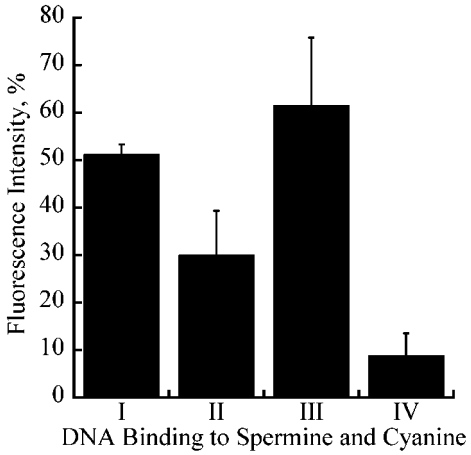

Fig. 4 Effects of addition sequence upon spermine inhibition of cyanine binding to genomic DNA. Binding reactions were with 1.13 fmol of DNA, 10 μM cyanine and spermine in 20 μL of buffer followed by dilution to 100 μL V_t as follows: I) DNA was mixed with cyanine, incubated for 10 min, followed by addition of 10 μM spermine and diluted in buffer; II) DNA was mixed with 10 μM spermine, incubated as above, followed by addition of cyanine and diluted in buffer; III) DNA was mixed with cyanine as above, then 0.5 μM spermine was added and diluted in methanol–water; IV) DNA was mixed with 0.5 μM spermine as above, then cyanine was added and diluted in methanol–water. Cyanine binding to DNA in the absence of spermine was considered as 100% for I and II (diluted with buffer) and III and IV (diluted with methanol–water), respectively.

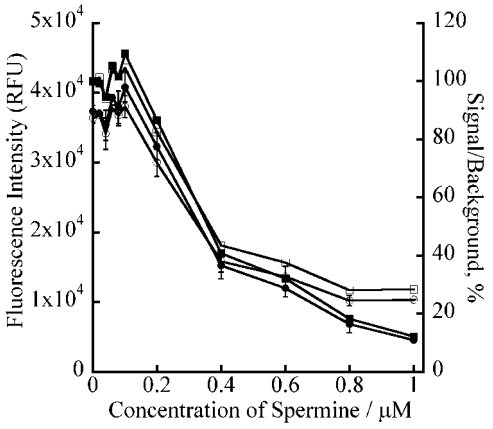

Fig. 5 IC_{50} for spermine inhibition of cyanine binding to genomic DNA. Increasing concentrations of spermine were added to 1.3 fmol of DNA in buffer and allowed to incubate for 10 min as described in Fig. 4. Then 10 μM cyanine (for 20 μL V_t) was added, followed by diluting the mixture to 100 μL with methanol–water. Circles represent fluorescence intensity and squares are *S/B*. Open symbols represent values obtained immediately after dilution and closed symbols values after 10 min post-dilution.

Table 1 Reversibility of DNA–model drug interactions by excess cyanine

Cyanine (μM) (20 μL V_t)[a]	% of control reactions (without spermine = 100%)		
	0.1 μM Spermine	0.25 μM Spermine	0.5 μM Spermine
20	59.3 ± 4.0[b]	35.1 ± 3.8	0
	(77.0 ± 8.4)[c]	(38.2 ± 6.3)	(0)
30	57.6 ± 2.6	55.0 ± 10.6	36.0 ± 5.0
	(80.5 ± 3.3)	(75.5 ± 8.3)	(100.0 ± 7.4)
40	63.3 ± 3.5	51.6 ± 0.7	56.5 ± 7.0
	(99.6 ± 5.4)	(85.7 ± 0.0)	(94.6 ± 9.7)

a. 20 μL V_t is the buffer volume during binding reactions consisting of 1.1 fmol of genomic DNA along with the indicated concentrations of spermine and cyanine. Reactions were diluted to 100 μL with methanol–water prior to fluorescence measurements.
b. Values outside parenthesis are fluorescence intensity calculations immediately after dilution with methanol–water.
c. Values inside parenthesis are fluorescence calculations from 10 min post methanol–water dilution.

ratio was only 0.05. Even with 20-fold lower spermine/cyanine ratio, the fluorescence decrease was more than double in methanol milieu relative to buffer. Thus, all the data were consistent.

During studies of DNA–model drug interactions, cyanine displacement could be carried out in two ways: allowing the dye to bind to DNA and examining the potency of the drug to dislodge the bound dye.[2,6] Alternately, the drug may bind to DNA first, followed by the dye displacing the bound drug. We conducted our DNA–model drug interactions both ways, although the latter yielded greater fluorescence diminishment. Here, DNA was first exposed to spermine, followed by cyanine addition, and finally diluting the reactions to 16% (v/v) methanol in 100 μL V_t.

IC_{50} and K_a

We refined the IC_{50} of spermine for DNA-cyanine J-aggregate and calculated it as 0.35 ± 0.03 μM spermine in methanol (Fig. 5). The inhibition profiles were similar when fluorescence was measured immediately after dilution or 10 min later. Repeatability and reproducibility were verified by similar IC_{50} values from experiments conducted more than 2 months apart. When the binding was carried out in buffer, the IC_{50} was 3.97 ± 0.47 μM spermine (>10-fold inhibitory potency due to methanol). The association constants (K_a) in methanol and buffer were estimated as 2.86×10^6 and 0.25×10^6 M^{-1}, respectively.

System reversibility

We calculated the cyanine concentration required to displace the DNA-bound polyamine (Table 1). The enhanced fluorescence recovery after 10 min was consistent with our previous data for optimal self-assembly.[7] It is clear that our DNA-model drug system was reversible since increasing concentrations of cyanine displaced spermine from DNA, reversing the fluorescence diminishment. For example, when spermine/cyanine ratio was 0.125 (Table 1, last column, first row), the fluorescence became extinguished. When this ratio declined to 0.08 and then to 0.0625, fluorescence was regained to nearly the same level as in the absence of spermine, demonstrating system reversibility.

System flexibility

We expanded our DNA–model drug interactions by substituting with λDNA. It is unclear regarding the 20 – 30% *S/B* increase in cyanine binding to λDNA with 0.1 to 0.2 μM spermine at 25 and 50 μM cyanine (Fig. 6). Nevertheless, the profiles highlighted several similarities between genomic DNA and λDNA interactions. Spermine produced a dose-dependent fluorescence decrease from λDNA-cyanine J-aggregate similar

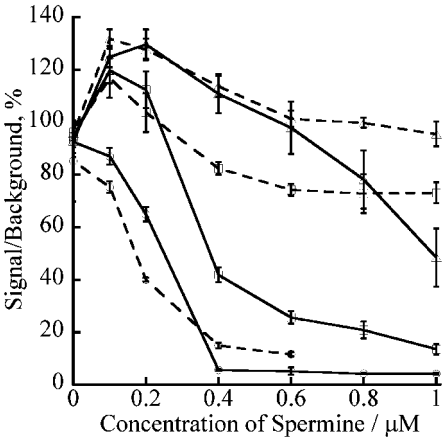

Fig. 6 Spermine inhibition of cyanine binding to λDNA. Indicated concentrations of spermine was bound to 4 fmol of DNA along with 10 μM (circles), 25 μM (squares) or 50 μM (triangles) of cyanine. Solid tracings represent DNA mixed with spermine, incubated for 10 min, followed by the addition of cyanine. Broken tracings represent reactions where cyanine was first self-assembled upon DNA, followed by displacement with spermine. Since fluorescence intensity varied with increasing concentrations of cyanine, the data was normalized as *% S/B*. The *S/B* in the absence of spermine was 100%.

to genomic DNA. Fluorescence drop was similarly higher in methanol relative to buffer. Like genomic DNA (Fig. 4), the drop was also higher when λDNA was first exposed to spermine followed by cyanine, confirming the importance of addition sequence (Fig. 6). For example, with 25 μM cyanine, when λDNA was bound to spermine first, the IC_{50} was 0.37 μM (Fig. 6). However, when cyanine was first allowed to self-assemble upon λDNA scaffold, the IC_{50} could not be calculated since the tracing became a flat line, parallel to the abscissa between 0.6 and 1.0 μM spermine. The IC_{50} for λDNA at 10 μM cyanine was 0.17 – 0.25 μM spermine, slightly more potent than genomic DNA. However, the IC_{50} of 0.37 μM spermine for λDNA with 25 μM cyanine (Fig. 6) is close to the 0.35 μM spermine IC_{50} calculated for genomic DNA with 10 μM cyanine (*vide supra*).

Figure 6 documented the inhibition of cyanine binding to λDNA and the reversal of inhibition by increasing concentrations of cyanine, similar to genomic DNA. For example, with prior exposure of λDNA to spermine, at 10 μM cyanine, the IC_{50} was 0.25 μM spermine. In the presence of 25 μM cyanine, the IC_{50} increased to 0.37 μM and increased further to 0.97 μM spermine when 50 μM self-assembled cyanine was displaced from the λDNA scaffold. Between 25 and 50 μM cyanine, the IC_{50} values of spermine dislodging the pre-assembled dye could not be calculated since the dose-response tracings became flat lines, parallel to the abscissa between 0.6 to 1.0 μM spermine. These data demonstrated the flexibility and reversibility of our DNA–model drug interactions under conditions of both pre- and post-exposure of genomic or λDNA to spermine or cyanine.

Discussion

Dye displacement for probing DNA–drug interactions have widely employed EB,[2,6,12-14] despite its toxic, carcinogenic, teratogenic and mutagenic properties.[8] Ethidium bromide has stringent considerations for safety, storage, handling, decontamination and disposal.[8] It requires UV illumination for visualizing the dye-stained DNA with a potential for accidental retinal damage, necessitating eye shields. Asymmetric or symmetric cyanines such as the chromophore shown in Fig. 1B are considered safer relative to EB.[8,15-18] We therefore explored a supramolecular self-assembling cyanine as a substitute for EB in HTS-compatible microwell reactions. The choice of cyanine was due to this dye forming intensely fluorescent J-aggregates on DNA scaffolds.[7] Our cyanine fluoresces under visible light, eliminating UV hazard. Cyanine, similar to DNA-binding drugs, interacts simultaneously with different DNA molecules *via* inter- and intra-molecular reactions.[9] Our studies with cyanine chemistry[7] might aid rational drug[4] and dye[19] design.

Spermine as a model DNA-binding drug was chosen for the following considerations. Polyamines such as spermine, spermidine, putrescine and cadaverine are cationic molecules present in millimolar concentrations in cells.[9,20,21] These compounds bind to a variety of macromolecules including DNA.[9,21] Among the polyamines, spermine efficiently binds and stabilizes DNA.[11] Spermine is the dominant polyamine in *Escherichia coli*,[9] the source of our DNA. In studies with EB, polyamines were used for DNA–model drug interactions or as potential gene delivery vehicles.[9,11,12,22-24] Similar to DNA–drug interactions, cyanines can intercalate or self-assemble upon DNA double helix.[7,10,25] Despite extensive studies, the exact mechanism of polyamines binding to DNA remains to be finalized;[9,21] our studies might aid in the further elucidation of these interactions. Finally, spermine-induced DNA condensation is useful for studying DNA packaging inside the nucleus.[26]

Normally, DNA exists as a right-handed double helix, the B-DNA; polyamine binding induces conformational changes such as conversion to left-handed Z-DNA. Polyamine binding results in DNA condensation and helix stabilization through electrostatic interactions between the cationic amino and the anionic phosphate groups, resulting in charge neutralization. Polyamines also interact with DNA *via* hydrophobic and van der Waal's forces, hydration and salt bridges.[9,20,26,27] Numerous factors regulate DNA–polyamine interactions including type, sequence, secondary structure, cooperative effects, temperature, Mg^{2+}, ionic strength, solvent milieu, surface geometry, charge, methylene and amino groups distributed along the polyamine backbone, charge and inter-charge distance. These factors contribute to delocalized and sequence-dependent binding of polyamines to DNA.[9,11,20,23,24,26,27]

Given the variety of factors influencing DNA–spermine interactions, the ability of our supramolecular self-assembling cyanine to displace the polyamine was remarkable. Dynamics of the formation and disassembly of the complex detected through fluorescence might arise from fluorescence quench by spermine or a competition between the polyamine and the dye for the same or similar binding sites on DNA. Mutual reversibility by polyamine (Figs. 1 – 5) or cyanine (Fig. 6, Table 1) makes ligand displacement the likely mechanism without excluding some degree of quench. Cyanine displacement is thus useful for studying DNA–drug interactions. Reciprocal of IC_{50} transforming into K_a[11,12,28,29] assumes that spermine and cyanine compete for the same/similar binding sites on DNA, like the widely employed EB.[2,6,12-14,22-24] However, this K_a is only an estimate, since affinity is influenced by several factors (*vide supra*), and we did not factor the K_a of cyanine for DNA during affinity calculations.[12,28,29] Our K_a of ~10^6 M^{-1} is within the 10^5 to 10^{11} M^{-1} range for DNA–drug interactions using EB[2] and similar to the 1.4×10^6 M^{-1} K_a for spermine binding to λDNA at 17 mM ionic strength[26] compared to the 20 mM NaCl we used. Our K_a is similar to the 5×10^6 M^{-1} calculated for spermine binding to calf thymus DNA using EB,[28] but differs

from the mutually exclusive affinity values of 2.3×10^5, 1.97×10^7 and 5.7×10^4 M^{-1} reported using the exact same reagents,[21,29,30] thereby echoing the difficulties in comparing literature K_a values.[31]

Alcohols lead to DNA condensation and ethanol is routinely used to precipitate DNA.[32] Ethanol, 2-propanol and *tert*-butanol caused calf thymus DNA to condense accompanied by ellipticity changes, similar to the polymer-and-salt induced ψ forms.[33] Thirty different solvents, including known helicogenics, were surveyed for solvent-induced equilibrium shifting between folded and unfolded states during foldamer research, analogous to biomacromolecules such as DNA, resulting in the helical conformation becoming more stabilized with increasing solvent polarity, providing there were no solvent–chain interactions.[34] We reported that 20% methanol was necessary and sufficient for maximal fluorescence from DNA-cyanine J-aggregates.[7] The 16.7% methanol potentiating spermine inhibition of cyanine binding approaches this limit (Fig. 2). Methanol exerts complex effects on DNA–model drug interactions in the presence of cyanine. Methanol enhances the rate and extent of DNA condensation by lowering the dielectric constant of the reaction milieu and exerts electrostatic and conformational effects on DNA, particularly under the low ionic strength conditions[26,32] of our studies. Methanol and polyamine thus act synergistically to condense the DNA[32] and the combined effects apparently overwhelm the enhancement effects of methanol on cyanine binding to DNA.[7] The data hints that cyanine binds poorly to collapsed and possibly left-handed DNA relative to the elongated helix, since DNA condensed by polyamine and methanol prevented cyanine binding. Reversal of inhibition in the presence of excess cyanine (Fig. 6, Table 1) implies favorable dye binding to relaxed, possibly right handed, elongated DNA following displacement of the primary condensing agent, *i.e.*, spermine. Under these conditions, methanol perhaps reverts to its role of enhancing cyanine binding to DNA.[7] Cyanine binding sites might be lost, inaccessible or camouflaged following DNA collapse by spermine and methanol.

Conclusions

We conducted DNA–model drug–dye displacement reactions in 384-well microplates with reaction volumes of 20 to 100 μL to demonstrate assay miniaturization and HTS capabilities,[5] unlike 3 mL used during EB displacement.[23,24] During HTS, the compound library might be dispensed in microwells containing DNA. The robotics/liquid handling system dispenses cyanine to the wells and fluorescence is measured for rapid analyses of DNA–drug interactions. The magnitude of fluorescence decrease was larger in this sequence (Figs. 4 and 6) although the cyanine may be bound to DNA and then displaced by the drug (Figs. 1A, 1B, 4 and 6). Spermine dissociation by cyanine leads to DNA de-condensation that could be a conformational probe for gene expression or shut off.[1] Our studies are broadly applicable since collapsed or relaxed DNA structures might vary with different ligands, the overall condensation mechanism is likely to be similar,[26] and can be studied in HTS format using supramolecular self-assembling cyanine. Studies are in progress to further investigate the macro-ions collapse, the binding sites of cyanine on nucleic acid scaffolds, solvent polarity and the polyamine chain length/chemistry, tuning the collapse.

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