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Macro-Ions Collapse Leading to Hybrid Bio-Nanomaterials

Komandoor Achyuthan

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Komandoor Achyuthan (Biosensors and Nanomaterials, 01714)

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Abstract

I used supramolecular self-assembling cyanine and the polyamine spermine binding to *Escherichia coli* genomic DNA as a model for DNA collapse during high throughput screening. Polyamine binding to DNA converts the normally right handed B-DNA into left handed Z-DNA conformation. Polyamine binding to DNA was inhibited by the supramolecular self-assembling cyanine. Self-assembly of cyanine upon DNA scaffold was likewise competitively inhibited by spermine as signaled by fluorescence quench from DNA-cyanine ensemble. Sequence of DNA exposure to cyanine or spermine was critical in determining the magnitude of fluorescence quench. Methanol potentiated spermine inhibition by >10-fold. The IC_{50} for spermine inhibition was 0.35 ± 0.03μM and the association constant *Ka* was 2.86 x 10^{-6}M. Reversibility of the DNA-polyamine interactions was evident from quench mitigation at higher concentrations of cyanine. System flexibility was demonstrated by similar spermine interactions with λDNA. The choices and rationale regarding the polyamine, the cyanine dye as well as the remarkable effects of methanol are discussed in detail. Cyanine might be a safer alternative to the mutagenic toxin ethidium bromide for investigating DNA-drug interactions. The combined actions of polyamines and alcohols mediate DNA collapse producing hybrid bio-nanomaterials with novel signaling properties that might be useful in biosensor applications. **Finally, this work will be submitted to Analytical Sciences (Japan) for publication.** This journal published our earlier, related work on cyanine supramolecular self-assembly upon a variety of nucleic acid scaffolds.

ACKNOWLEDGMENTS

I thank Drs. Stephen Caslanuovo and Wahid Hermina for project support. Prof. David Whitten supplied the cyanine that was used in these studies. Dr. Supratim Datta (Org # 08625) performed the Center 1700-mandated peer review.

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NOMENCLATURE

DOE	Department of Energy
HTS	High-throughput screening
LDRD	Laboratory Directed Research and Development
SNL	Sandia National Laboratories

1.0 INTRODUCTION

Consider that the total length of human DNA is approximately 140 astronomical units or about 700 round trips between the Earth and the Sun, yet it is packed inside cells that are only 10 microns in size. Each human cell contains DNA approximately 3 meters in length. Clearly, biological systems have figured out how to use higher-order structures (self-assembled folding) to optimize the properties and function of biomolecules such as DNA and proteins. An understanding of these processes will provide significant insight and direction to the formation of synthetic nanomaterials. Hybrid nanomaterials with synthetic and natural molecules offer new properties absent in their components; however, the design and control of such nanohybrids has proved daunting. Self-assembly of DNA macro-ions and synthetic materials to form useful hybrid bio-nanomaterials is therefore a formidable challenge. The theoretical understanding of reversible complexation/de-comlexation transitions could be useful for understanding the assembly of more complex structures in controlled environments at a macro level.

Drugs may be developed to treat diseases by interfering with the main functions of DNA, transcription and replication.[1] Thus, DNA-drug interactions are key 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] A variety of techniques were used to study DNA-drug interactions including gel shift, filter binding, nuclear magnetic resonance (NMR), Raman spectroscopy, mass spectrometry (MS), calorimetry, surface plasmon resonance (SPR), dialysis, ultrafiltration, electrophoresis, ultracentrifugation, high performance or thin layer chromatography (HPLC, TLC) and the optical techniques of absorption and fluorescence.[2-4] Most of these techniques are time intensive or require sophisticated equipment and/or trained operators. Several are not amenable to high throughput screening (HTS), essential in drug discovery.[5]

One method to screen DNA-drug interactions is the “dye displacement” assay.[6] Here, a non-fluorescent dye becomes fluorescent upon binding to DNA. Displacement of the dye from DNA by a drug leads to fluorescence quench, signaling DNA-drug interactions. The widely used ethidium bromide (EB)[2,6] dye is mutagenic.[7] As an alternative to EB, I report a cyanine dye that spontaneously self-assembles on DNA scaffold accompanied by bright fluorescence emission from the J-aggregate.[8] I used DNA-spermine binding[9] as a model for DNA-drug interactions.[2-4] I describe the various factors influencing the competition between cyanine and polyamine for binding to DNA and a ~10-fold potentiating effect of methanol on fluorescence quench. By carrying out the DNA-model drug interactions in 384-well microplates I demonstrated the capabilities for assay miniaturization, robotics, liquid handling and HTS.[5]

There is a need for the sensitive detection of chem.-bio agents for military and national security applications. My studies might enable the prediction of how nanomaterials organize, self-assemble and function, based upon the molecular properties of the constituent components. In this effort I took a page out of nature for the study of self-assembling nanomaterials. This is a key step toward end-use driven designer nanomaterials and aligns with the NTM mission to “control and manipulate” matter “to attain unique properties and function.”

2.0 Experimental

2.1 Reagents and chemicals

Escherichia coli genomic DNA was from Sigma Aldrich (St. Louis, MO) and λDNA from Promega (Madison, WI). Details regarding these reagents can be found in my earlier publication.[8] The polyamine spermine [$C_{10}H_{26}N_4$] (98% pure; moisture, 0.33%; *Mr* = 202.3) was purchased from MP Biomedicals (Solon, OH). The FTIR spectrum of spermine conformed to the standard. Spermine was dissolved in water as a 797mM stock solution and stored in small aliquots at –20^{o}C. Cyanine solution was prepared and used in DNA binding assays as described previously.[8] All binding experiments were conducted in phosphate buffer (2mM sodium phosphate, pH 7.5, 20mM NaCl and 10μM ethylenediamine tetraacetic acid) similar to a buffer described previously.[10]

2.2 DNA binding assay

The binding reaction consisted of DNA, cyanine and/or polyamine in a total volume (*Vt*) of 20μL buffer. The reaction mixture was agitated to ensure uniform mixing using the "automix" function of the software drive microplate reader (Molecular Devices M2, Sunnyvale, CA). All reactions were carried out at ~25^{o}C using freshly diluted reagents in buffer. After 10minutes incubation, the reactions were diluted to 100μL *Vt* using either buffer or methanol-H_2O mixture (20:80, v/v), before taking fluorescence measurements. Volume changes due to addition of various reagents were less than 10%; therefore, no corrective calculations were made in accordance with previously established practice.[11] Reactions were carried out in 384-well white microplates (Optiplate, Perkin Elmer, Waltham, MA). Various "control" reactions were done simultaneously including buffer only, DNA only, cyanine only, polyamine only and DNA+polyamine along with DNA+cyanine (100% control)[11] and "complete" reaction (DNA+polyamine+cyanine). None of the control reactions yielded fluorescence emission greater than 10% of maximal fluorescence from the DNA+cyanine supramolecular ensemble. These "control" reactions were used to correct the fluorescence from DNA-cyanine J-aggregate. Samples were excited at 425nm and fluorescence emission was measured at 470nm.[8] In one experiment (Fig. 1B), 450nm excitation wavelength was used. Emission intensity was expressed as relative fluorescence units (RFU).

2.3 Data analysis

Data were collected in triplicate and the results expressed as average ± standard deviation. Where not visible, error bars are masked within the symbol. Charts were created using Kaleidagraph (Synergy Software, Reading, PA). The concentration of polyamine to cause an inhibition of cyanine binding by one-half as reflected by a 50% quench of the fluorescence from the DNA-cyanine J-aggregate was designated as IC_{50}. The association (binding) constant (*Ka*) was calculated from the reciprocal of the IC_{50} value.[11,12] In addition to the difference between the signal *minus* background (S – B), the data was also calculated as signal divided by background (S/B). To simplify visualization and facilitate side-by-side comparisons of different experimental data, graphs were also depicted as percent changes to fluorescence intensity or S/B.

3.0 RESULTS

3.1 Polyamine inhibition of cyanine binding to DNA

Increasing concentrations of spermine produced a dose-dependent fluorescence quench indicative of a competitive interaction with cyanine for binding to DNA. These results were similar when the reaction mixtures were examined at two different excitation wavelengths (Figs. 1A and 1B). The inhibition profiles were also similar regardless of whether the data was analyzed as S – B or as S/B at either wavelength (compare Figs. 1A and 1B). For example, the *preliminary* IC_{50} values in methanol-water were 3.0 and 4.0μM respectively, when analyzed as S – B and S/B at 425nm excitation wavelength (Fig. 1A). Likewise, the *preliminary* IC_{50} was 3.3μM (S – B) at 450nm excitation wavelength (Fig. 1B). Depending on particular applications, the reactions may be monitored for lower overall RFU and higher S/B by exciting the samples at 425nm (Fig. 1A). Conversely, if higher RFU was desired, then 450nm excitation wavelength may be used (Fig. 1B). These results were reproducible when the experiment was repeated after a 24 hour interval demonstrating repeatability. Another important finding of the data of Fig. 1 was that the addition of methanol enhanced spermine-induced fluorescence quench. On the other hand, even though spermine produced a dose-dependent modest fluorescence quench and moderate decrease to S/B in the absence of methanol, the inhibition did not reach 50% level even with 10μM spermine regardless of the excitation wavelength (Figs. 1A and 1B).

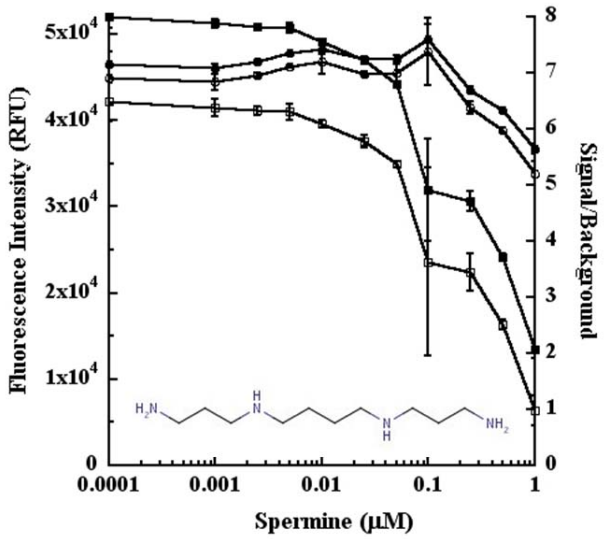

Fig. 1A: Spermine inhibition of cyanine binding to Escherichia coli genomic DNA using 425nm excitation wavelength. Increasing and indicated concentrations of spermine were mixed with 1.2fmol of DNA and 10μM cyanine in 20μL buffer. Fluorescence was measured at 470nm. The open symbols represent fluorescence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

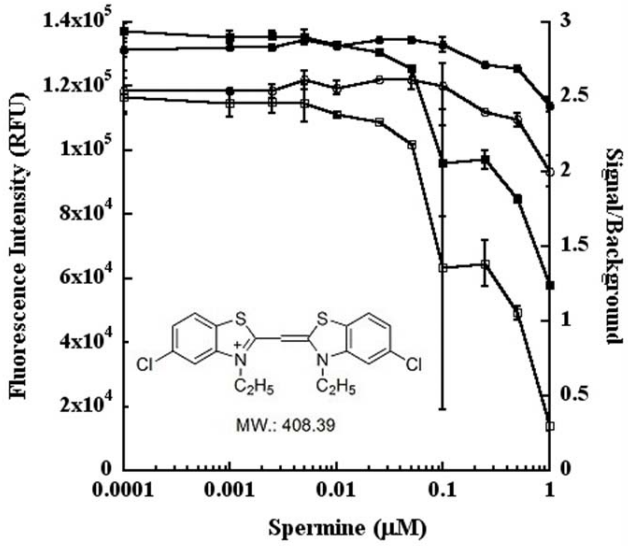

Fig. 1B: **Spermine inhibition of cyanine binding to DNA using 450nm excitation wavelength.** The reactions from Fig. 1A were interrogated using 450nm excitation wavelength. All other conditions are the same as those described for Fig. 1A. The inset shows the structure of cyanine.

3.2 Methanol dilution effects

I next investigated in detail the effects of methanol on the competitive inhibition of cyanine binding to DNA by spermine. Here, the concentration of DNA, cyanine and spermine were fixed during the binding segment of the experiment. Subsequent dilution of the reactions by adding increasing volumes of methanol-water mixture led to progressively declining molar concentrations of all three reagents even though the *amounts* of the reagents were not altered. For example, when the reaction volume was 20μL *Vt* in the absence of methanol, cyanine concentration was 10μM and spermine concentrations ranged from 0.1 to 100μM. When the well volume reached 100μL *Vt* due to incremental additions of methanol-water, cyanine concentration dropped to 2μM and spermine concentrations ranged from 0.02 to 20μM due to 5-fold dilution. However, the ratio of spermine/cyanine did not change over the entire dilution range. I therefore profiled the data either as fluorescence quench (Fig. 2A) or decrease in the normalized percent fluorescence (Fig. 2B) as a function of increasing spermine/cyanine ratios. Volumes larger than 100μL *Vt* were not attempted since maximal effect was observed between 16 and 16.7% (v/v) methanol and also because the 384-microplate well capacity was reached.

Consistent with the data of Figs. 1A and 1B, there was indeed a methanol dose-dependent increase in the fluorescence quench with maximal quench at ~16% (v/v) methanol (Figs. 2A and 2B). Due to increasing dilutions, overall fluorescence intensity *in the absence of spermine* decreased from 47085 ± 5586 RFU at 0% methanol (20μL *Vt*) to 32170 ± 3677 RFU in 16% (v/v) methanol concentration (100μL *Vt*), representing a 31.7% drop in fluorescence intensity

due to dilution. For this reason, I transformed the data as normalized percent changes to the fluorescence intensity (Fig. 2B). The inhibition profiles were however similar regardless of how the data was analyzed (compare Figs. 2A and 2B). For example, 50% fluorescence quench was obtained when the spermine/cyanine ratio reached 0.10 at 16% (v/v) methanol (Fig. 2A). Likewise, one-half decline in percent fluorescence was observed at a spermine/cyanine ratio of 0.09 (Fig. 2B). Furthermore, in both types of analyses, IC_{50} values could not be calculated between methanol concentrations of 0 to 6.7% (v/v), confirming the similarity of the data in Figs. 2A and 2B. In order to simplify data visualization and to compare different experimental results, I charted subsequent results as changes to percent fluorescence or percent S/B.

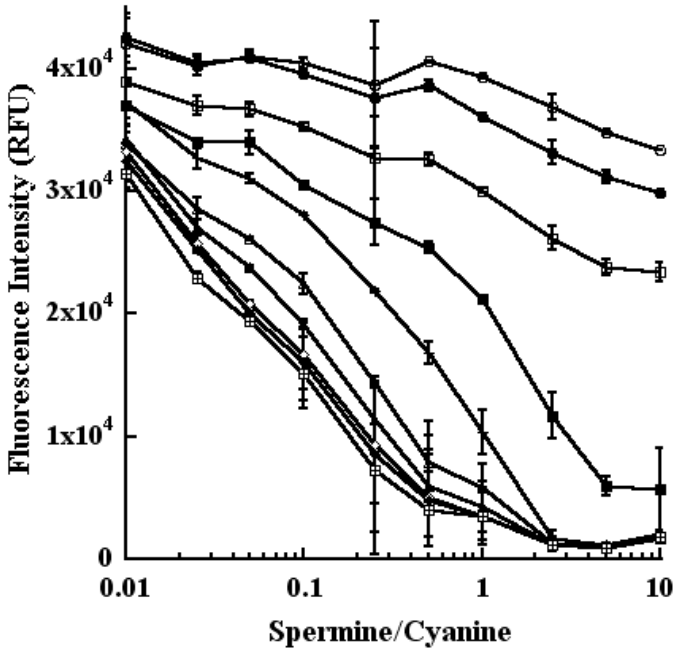

Fig. 2A: Effect of methanol on spermine inhibition of cyanine binding to *Escherichia coli* genomic DNA monitored by changes to the fluorescence intensity. Increasing concentrations of spermine were mixed with 1.3fmol of DNA and 10μM cyanine dye in 20μL *Vt*. The reaction wells were then diluted with increasing volumes of methanol-water mixture and measuring the fluorescence intensity after each incremental addition of the solvent. The tracings represent fluorescence signal 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%; and open square enclosing plus sign, 16%. Simultaneous with the increase in methanol concentrations, decrease to cyanine concentrations due to dilutions were as follows (μM): 10, 8, 6.7, 5, 4, 3.33, 2.85, 2.5, 2.22 and 2.0.

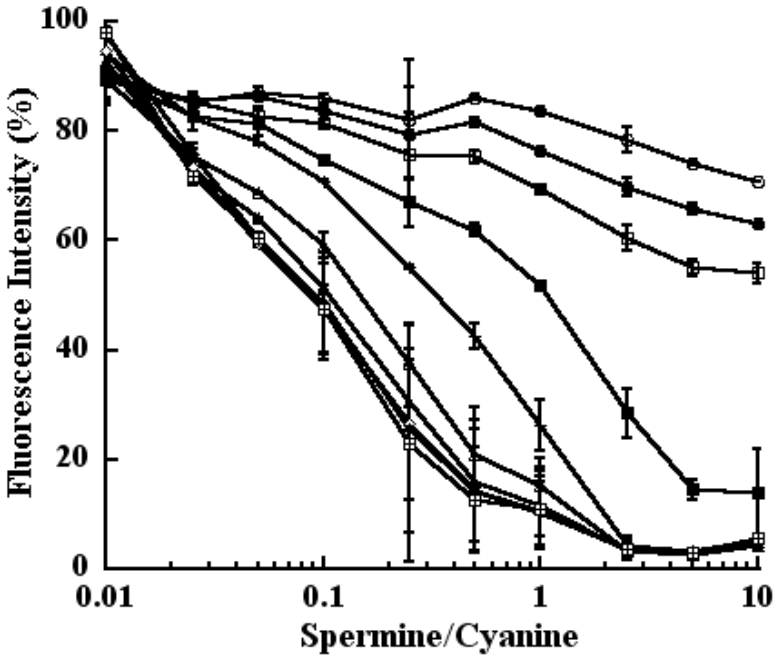

Fig. 2B: Effect of methanol on spermine inhibition of cyanine binding to DNA by changes to the normalized percent fluorescence emission. All conditions are as described for Fig. 2A except that the fluorescence intensity was transformed into percent values with emission in the absence of spermine being 100%.

3.3 Buffer dilution effects

In order to exclude the possibility that the enhanced fluorescence quench was related to the dilution and not specific to methanol, I tested the effects of adding equivalent volumes of buffer instead of methanol-water (Fig. 3). The exact same incremental additions of buffer were used here as the incremental volumes of methanol-water added in Fig. 2. Consequently, the spermine/cyanine ratios varied identically in both sets of experiments. Thus, except for the nature of diluent (methanol-water *versus* buffer), all other reaction conditions were identical in both experiments. It is clear that methanol indeed enhanced the fluorescence quench by spermine greater than 10-fold, since 50% fluorescence quench was observed at a spermine/cyanine ratio of 1.3 in buffer (Fig. 3) relative to spermine/cyanine ratio of 0.09 in 16% (v/v) methanol (Fig. 2B). Similarly, at 10% (v/v) methanol, IC_{50} was reached when the spermine/cyanine ratio was 1.0 (Fig. 2B). By contrast, for the same dilution in buffer, IC_{50} was at a spermine/cyanine ratio of 10.0 (Fig. 3). These data confirmed the specific potentiating effect of methanol upon spermine inhibition of cyanine binding to DNA.

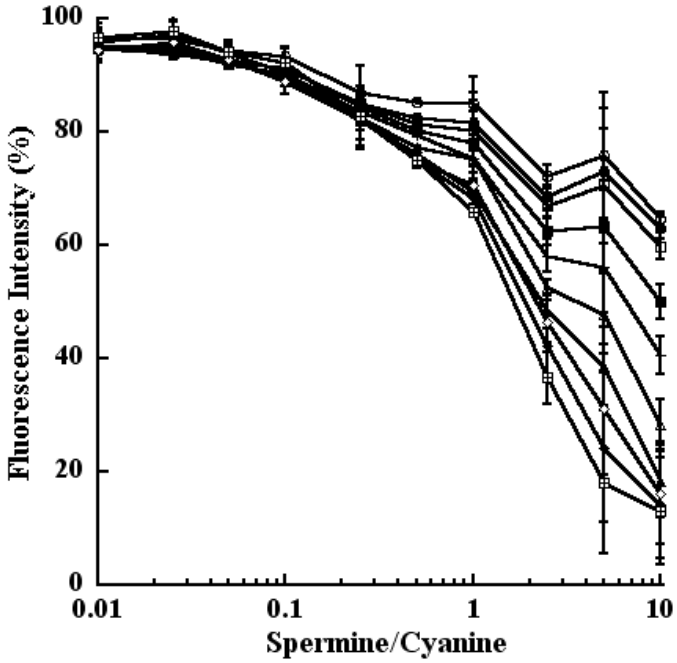

Fig. 3: Effect of buffer on spermine inhibition of cyanine binding to *Escherichia coli* genomic DNA. All conditions are *exactly* the same as described for Fig. 2A, except that incremental additions of buffer was used to dilute the binding reactions instead of methanol-water mixture. Consequently, the tracings represent progressively diminishing concentrations (μM) of cyanine similar 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.

3.4 Addition sequence

I then tested the effects of exposing the DNA to polyamine first, incubating for 10 minutes followed by the addition of cyanine or reversing that order by exposing the DNA to the cyanine first followed by the addition of spermine. As seen in Fig. 4, prior exposure of DNA to spermine significantly enhanced the cyanine J-aggregate fluorescence quench. These results were obtained regardless of whether the reaction was diluted in buffer or methanol-water mixture (Fig. 4). In agreement with the data of Figs. 1 – 3, greater fluorescence quench was also observed by diluting the reaction with methanol-water instead of buffer. Thus, when the reaction was diluted using buffer upon prior exposure to DNA, spermine enhanced the overall quench by 20% (from 50% to 30% residual fluorescence). On the other hand, dilution using methanol-water resulted in an enhanced quench of 52% (from 60% to 8% residual fluorescence) (Fig. 4). This is even more remarkable considering the spermine/cyanine during buffer dilution was 1.0 whereas in methanol-water dilution, that ratio was only 0.05. Even with 20-fold smaller spermine/cyanine ratio, fluorescence quench was more than double in methanol milieu relative to buffer. Thus, all the data were internally consistent.

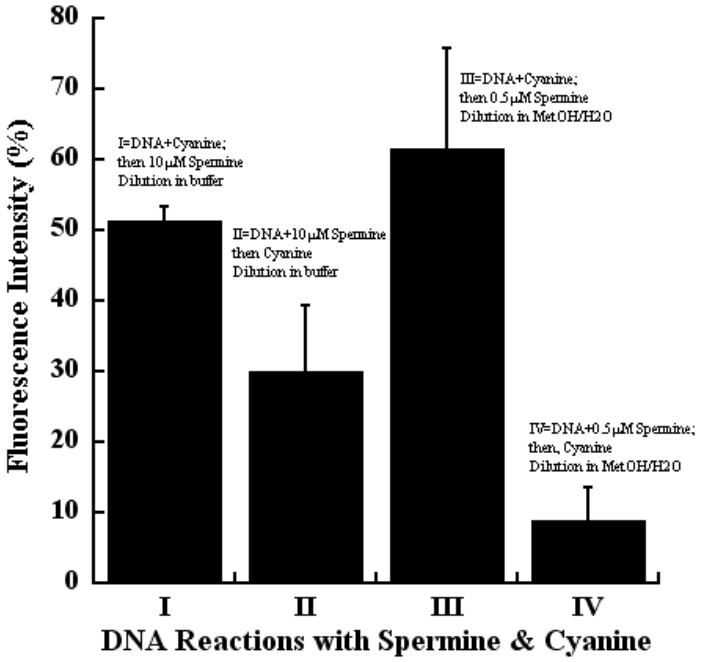

Fig. 4: Effect of reagents addition sequence upon spermine inhibition of cyanine binding to *Escherichia coli* genomic DNA. Binding reactions were carried out using 1.13fmol of DNA, 10μM cyanine and the indicated concentrations of spermine in 20μL of buffer. The DNA was mixed first with spermine or cyanine, incubated for 10 minutes followed by the addition of the other reagent (cyanine or spermine). The reaction volume was then diluted to 100μL using either methanol-water mixture or buffer. Reactions of DNA with cyanine in the absence of spermine and diluted using methanol-water or buffer were considered as 100%.

During studies of DNA-model drug interactions, cyanine displacement could be carried out in two ways: allowing the dye to bind to DNA and then examining the potency of the drug to dislodge the bound dye. Alternately, the drug may bind to DNA first followed by the dye displacing bound drug. In view of the higher fluorescence quench, I conducted the model DNA-drug interactions using the former approach. All subsequent reactions were carried out by exposing the DNA to spermine first, followed by the addition of cyanine dye and finally diluting the reactions using methanol-water mixture to 16% (v/v) methanol in 100μL *Vt*.

3.5 IC_{50} and affinity constant

I next refined the spermine IC_{50} for the DNA-cyanine J-aggregates (Fig. 5). The IC_{50} was calculated as 0.35 ± 0.03μM spermine. In the absence of methanol, when the binding was carried out in an equivalent volume of buffer, the IC_{50} was 3.97 ± 0.47μM spermine, leading to a 11-fold increase in inhibitory potency due to methanol. The inhibitory profiles were similar when fluorescence was measured immediately after dilution or 10minutes after diluting with methanol. Repeatability and reproducibility were verified by similar IC_{50} values from experiments conducted more than 2 months apart. The association (binding) constant *Ka* in methanol was estimated as 2.86×10^{-6}M. The *Ka* in buffer was 0.25×10^{-6}M.

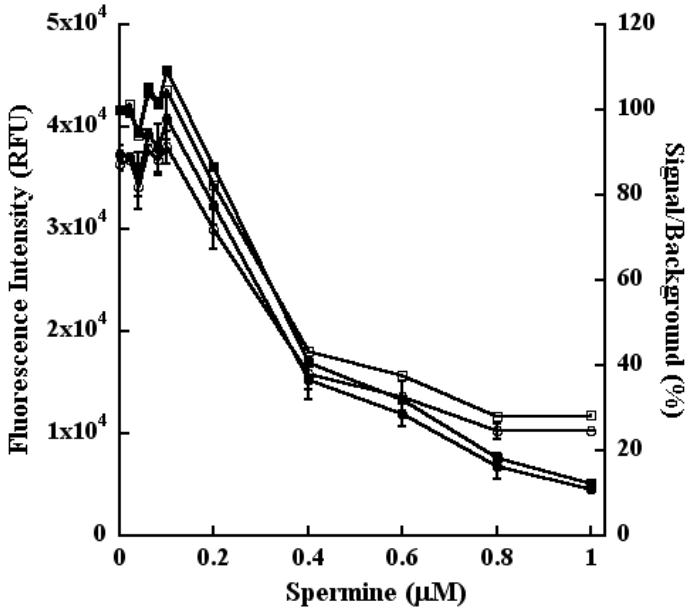

Fig. 5: IC_{50} determination for spermine inhibition of cyanine binding to *Escherichia coli* genomic DNA. Indicated and increasing concentrations of spermine were added to 1.3fmol of DNA in buffer and allowed to incubate for 10 minutes as described in the legend to Fig. 4. Then 10μM cyanine (for 20μL *Vt*) was added, followed by diluting the binding mixture to 100μL with methanol-water. The circles represent fluorescence intensities and squares are S/B. Open symbols are values obtained immediately after dilution and closed symbols represent values from 10minutes post-dilution incubation.

3.6 Reversibility

I then estimated the concentration of cyanine required to displace the DNA-bound polyamine (Table 1). The enhanced fluorescence recovery after 10 minutes is consistent with my previous data regarding a 15 minute time interval for optimal self-assembly.[8] It is clear that this DNA-model drug system was fully reversible since increasing concentrations of cyanine displaced the spermine from DNA reversing the fluorescence quench and regaining fluorescence emission. For example, when the spermine/cyanine ratio was 0.125 (last column, first row values, Table 1), there was 100% fluorescence quench. When this ratio declined to 0.08 and then to 0.0625, fluorescence emission was regained to nearly the same levels as in the absence of spermine, demonstrating system reversibility.

Table 1: Reversibility of DNA-model drug interactions by excess cyanine

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

*20μL *Vt* is the buffer volume during binding reactions consisting of 1.1fmol of *Escherichia coli* genomic DNA along with the indicated concentrations of spermine and cyanine. The reactions were diluted to 100μL with 80μL of methanol-water (20:80, v/v) just prior to fluorescence measurements.

**Values outside parenthesis were calculated from the fluorescence intensity values obtained from measurements immediately following dilution with methanol-water mixture.

***Values inside parenthesis were calculated from the fluorescence intensity values obtained from 10 minutes post methanol-water dilution.

3.7 System flexibility

I finally established that this DNA-model drug interactions were not limited to *Escherichia coli* genomic DNA by substituting with λDNA (Fig. 6). The profiles highlighted several similarities between the two types of DNA-model drug interactions. Spermine produced a dose-dependent fluorescence quench from the λDNA-cyanine J-aggregate similar to genomic DNA-cyanine J-aggregate. The fluorescence quench was higher in methanol relative to buffer. The quench was also higher when λDNA was first exposed to spermine followed by cyanine. For example, with 25μM cyanine, when λDNA was bound to spermine first, the IC_{50} was 0.37μM (Fig. 6). However, when the cyanine was *first* allowed to self-assemble upon λDNA scaffold, the IC_{50} could not be calculated since the tracing became a flat line running parallel to the abscissa between 0.6 and 1.0μM spermine. The IC_{50} for λDNA at 10μM cyanine was 0.17 – 0.25μM spermine, slightly more potent than the values calculated for genomic DNA. However, the IC_{50} of 0.37μM with 25μM cyanine for λDNA (Fig. 6) is close to the 0.35μM calculated for *E. coli* genomic DNA obtained with 10μM cyanine (*vide supra*).

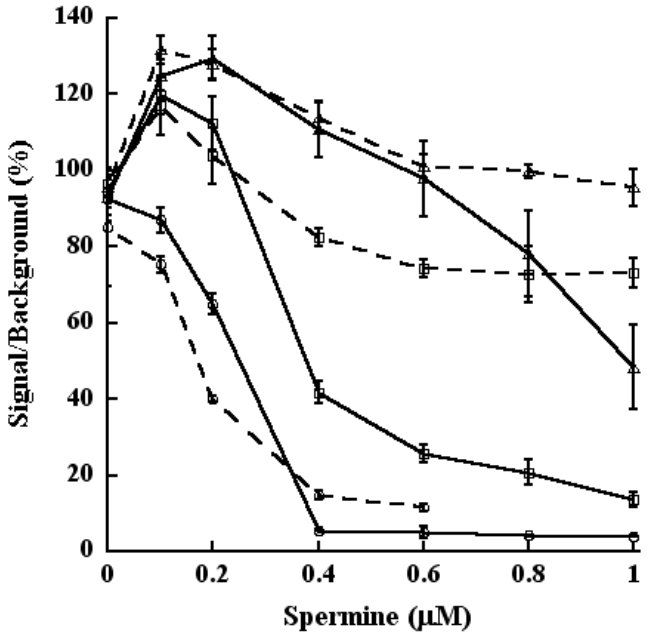

Fig. 6: Spermine inhibition of cyanine binding to λDNA. Increasing and indicated concentrations of spermine was bound to 4fmol of DNA along with 10μM (circles), 25μM (squares) or 50μM (triangles) of cyanine. Solid tracings are binding reactions where the DNA was first mixed with spermine, incubated for 10minutes followed by the addition of cyanine. Broken tracings represent binding reactions where cyanine was first self-assembled upon DNA scaffold followed by reaction with spermine. Since absolute fluorescence emission intensities varied with increasing concentrations of cyanine, the data was normalized as percent S/B with S/B in the absence of spermine being 100%.

Similar to genomic DNA, increasing concentrations of cyanine were able to reverse spermine inhibition with λDNA. For example, with prior exposure of λDNA to spermine, at 10μM cyanine the IC_{50} was 0.25μM spermine (Fig. 6). In the presence of 25μM cyanine, the IC_{50} increased to 0.37μM and increased further to 0.97μM spermine when 50μM of self-assembled cyanine was displaced from λDNA scaffold (Fig. 6). Between 25μM and 50μM cyanine, the IC_{50} values of spermine to dislodge the pre-assembled dye could not be calculated since the dose-response tracings were flat lines running parallel to the abscissa between 0.6 to 1.0μM spermine (Fig. 6). These data demonstrated the reversibility of the DNA-model drug interactions under conditions of both pre- and post-exposure of genomic or λ DNA to spermine or cyanine.

4.0 Discussion

Dye displacement for probing DNA-drug interactions have widely employed EB[2,6,13,14] despite its toxic, carcinogenic, teratogenic and mutagenic properties.[7] One study even utilized the mutagenic EB to study the binding of another mutagen, fecapentene, to DNA.[12] The EB dye has stringent safety considerations for storage, handling, decontamination, waste disposal and accidental spill cleanups.[7] EB requires UV illumination for visualizing the dye-stained DNA with a potential for accidental retinal damage, requiring protective eye shield.

Asymmetric cyanine dyes such as the chromophore shown in Fig. 1B are considered safer relative to EB.[7,15-18] I therefore explored supramolecular self-assembling cyanine as a substitute for EB. The choice of cyanine was based on prior experience with this dye forming intensely fluorescent J-aggregates on DNA scaffolds.[8] This cyanine fluoresces under visible light, eliminating workplace UV hazards. Cyanine shares an important and desirable property of DNA-binding drugs by interacting simultaneously with different DNA molecules *via* inter- and intra-molecular reactions.[9] Our studies with cyanine chemistries[8] might also aid rational drug design[4] as well as rational dye design.[19]

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 inside cells.[9,20,21] These compounds bind to a variety of macromolecules including DNA.[9,21] Among the polyamines, spermine is highly efficient in binding and stabilizing DNA.[11] It is also a predominant polyamine in *Escherichia coli,*[9] the DNA source. 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 on DNA double helix.[8,25] Despite extensive studies, the exact mechanism of polyamines binding to DNA remains to be finalized;[9,21] my studies might aid in the further elucidation of these interactions. Finally, beyond DNA-drug model, spermine-induced DNA condensation is useful to study DNA packaging processes inside the nucleus.[26]

Normally, DNA exists as a right-handed double helix, the B-DNA; polyamines binding to DNA induce conformationals change such as conversion to left-handed Z-DNA. Polyamine binding leads to DNA condensation and helix stabilization and involves electrostatic interactions between the cationic amino and the anionic phosphate groups leading to charge neutralization. Polyamines also interact with DNA *via* hydrophobic, van der Waal's forces, hydration and salt bridges.[9,20,26,27] Several factors regulate DNA-spermine interactions including sequence, reaction milieu, charge distribution along the surface of polyamine, the number and distribution of the methylene groups along the polyamine chain, geometry, the number and distribution of the amino groups along its backbone and the distance between these charges. These factors lead to both delocalized and sequence-dependent binding of polyamines to DNA. [9,11,20,26,27]

Given the variety of factors influencing DNA-spermine interactions, the ability of our supramolecular self-assembling cyanine to displace polyamine is remarkable. Dynamics of the formation and disassembly of the trimeric complex detected by fluorescence changes might arise either from emission quench by the polyamine or 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 a small extent of fluorescence quench. Cyanine displacement is thus a useful technique for studying DNA-model drug interactions. My calculations assume that spermine and cyanine compete for the same or similar binding sites on DNA. Spermine *Ka* is a relative binding affinity and probably overestimated as observed for fecapentene,[12] since affinity is influenced by the factors listed above along with temperature and ionic strength of binding reactions,[11,23,24] making it difficult to compare literature values.[28] Nevertheless, the *Ka* is similar to μM *Ka* (or *Kd*) reported previously for spermine binding to DNA in a low salt environment.[29]

Methanol exerts complex effects upon DNA-model drug interactions in the presence of cyanine. I and my colleagues reported that 20% (v/v) methanol was both necessary and sufficient for maximal fluorescence from DNA-cyanine J-aggregate.[8] The 16% (v/v) methanol used here is within this limit (Fig. 2). However, methanol also enhances the rate and extent of DNA condensation by lowering the dielectric constant of the reaction milieu and exerting electrostatic and conformational effects on DNA particularly under low ionic strength conditions,[30] similar to this study. Methanol and polyamine thus act synergistically to condense DNA[30] and the combined effects are apparently sufficient to overcome the enhancement effects of methanol on cyanine binding to DNA.[8] The data also suggests that cyanine does not bind well to collapsed and possibly left-handed DNA relative to the elongated helix, since DNA condensed by the actions of polyamine and methanol inhibited cyanine binding. Reversal of fluorescence quench in the presence of excess cyanine (Fig. 6, Table 1) implies favorable dye binding to relaxed, possibly right handed DNA following the displacement of the primary condensing agent, i.e., spermine. Under these conditions, it is likely that methanol reverts to its role of enhancing cyanine binding to DNA.[8] Cyanine binding sites might be lost, inaccessible or camouflaged following DNA collapse by spermine and methanol.

5.0 Concluding Remarks

In conclusion, I executed DNA-model drug-dye displacement studies using 384-well microplates in order to demonstrate assay miniaturization and HTS capabilities.[5] Spermine dissociation by cyanine leads to DNA de-condensation that could be a conformational probe for studies of gene expression or shut off.[1] My studies are broadly applicable since although collapsed or relaxed DNA structures might be different for different ligands, the overall condensation mechanism is likely to be similar[26] and can be studied in a HTS environment using supramolecular self-assembling cyanine. Studies are in progress to explore the reciprocal binding sites on different types of DNA scaffolds for cyanine and the various polyamines of spermine, spermidine, putrescine and cadaverine in order to further elucidate the macro-ion collapse.

This LDRD project made significant progress and reached important milestones. These were as follows: 1) Substitution of a safer, supramolecular self-assembling cyanine as an alternative to the mutagenic ethidium bromide in DNA-model drug interactions; 2) Identification of dual, opposing effects of methanol upon DNA-model drug dye displacement reactions by synergistically enhancing with polyamine the DNA collapse and maximizing cyanine self-assembly upon nucleic acid scaffolds; 3) High throughput screening (HTS) formatting of DNA-model drug interactions; 4) System reversibility and flexibility by demonstration applications with two different types of DNA (genomic and λ); ***5) This work will be submitted to Analytical Sciences (Japan) for publication.***

My studies might enable the prediction of how nanomaterials organize, self-assemble and function, based upon the molecular properties of the constituent components. In this effort I took a page out of nature for the study of self-assembling nanomaterials. This is a key step toward end-use driven designer nanomaterials and aligns with the NTM mission to “control and manipulate” matter “to attain unique properties and function.”

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