

SANDIA REPORT

SAND2009-5749

Unlimited Release

Printed October, 2009

Macro-Ions Collapse Leading to Hybrid Bio-Nanomaterials

Komandoor Achyuthan

Prepared by
Sandia National Laboratories
Albuquerque, New Mexico 87185 and Livermore, California 94550

Sandia is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy's National Nuclear Security Administration under Contract DE-AC04-94AL85000.

Approved for public release; further dissemination unlimited.



Sandia National Laboratories

Issued by Sandia National Laboratories, operated for the United States Department of Energy by Sandia Corporation.

NOTICE: This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government, nor any agency thereof, nor any of their employees, nor any of their contractors, subcontractors, or their employees, make any warranty, express or implied, or assume any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represent that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government, any agency thereof, or any of their contractors or subcontractors. The views and opinions expressed herein do not necessarily state or reflect those of the United States Government, any agency thereof, or any of their contractors.

Printed in the United States of America. This report has been reproduced directly from the best available copy.

Available to DOE and DOE contractors from
U.S. Department of Energy
Office of Scientific and Technical Information
P.O. Box 62
Oak Ridge, TN 37831

Telephone: (865) 576-8401
Facsimile: (865) 576-5728
E-Mail: reports@adonis.osti.gov
Online ordering: <http://www.osti.gov/bridge>

Available to the public from
U.S. Department of Commerce
National Technical Information Service
5285 Port Royal Rd.
Springfield, VA 22161

Telephone: (800) 553-6847
Facsimile: (703) 605-6900
E-Mail: orders@ntis.fedworld.gov
Online order: <http://www.ntis.gov/help/ordermethods.asp?loc=7-4-0#online>



SAND-2009-5749

Unlimited Release

Printed October 2009

Macro-Ions Collapse Leading to Hybrid Bio-Nanomaterials

Komandoor Achyuthan (Biosensors and Nanomaterials, 01714)

Sandia National Laboratories P.O. Box 5800 Albuquerque, New Mexico 87185-MS1080

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 $0.35 \pm 0.03 \mu\text{M}$ and the association constant K_a was $2.86 \times 10^{-6} \text{M}$. 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 λ DNA. 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.

Sandia National Laboratories is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy under contract DE-ACO4-94-AL85000.

CONTENTS

1. Introduction.....	7
2. Experimental.....	8
2.1 Reagents and chemicals	8
2.2 DNA binding assay	8
2.3 Data analysis	8
3. Results.....	9
3.1 Polyamine inhibition of cyanine binding to DNA	9
3.2 Methanol dilution effects	10
3.3 Buffer dilution effects	12
3.4 Addition sequence	13
3.5 IC ₅₀ and affinity constant	14
3.6 Reversibility	15
3.7 System flexibility	16
4. Discussion.....	18
5. Concluding Remarks.....	20
6. References.....	21
7. Distribution.....	22

FIGURES

Figure-1A: Spermine inhibition of cyanine binding to <i>Escherichia coli</i> genomic DNA using 425nm excitation wavelength	9
Figure-1B: Spermine inhibition of cyanine binding to DNA using 450nm excitation wavelength.	10
Figure-2A: Effect of methanol on spermine inhibition of cyanine binding to <i>E. coli</i> genomic DNA monitored by changes to the fluorescence intensity	11
Figures-2B: Effect of methanol on spermine inhibition of cyanine binding to DNA by changes to the normalized percent fluorescence emission	12
Figures-3: Effect of buffer on spermine inhibition of cyanine binding to <i>E. coli</i> genomic DNA	13
Figures-4: Effect of reagents addition sequence upon spermine inhibition of cyanine binding to <i>Escherichia coli</i> genomic DNA	14
Figure-5: IC ₅₀ determination for spermine inhibition of cyanine binding to <i>E. coli</i> genomic DNA	15
Figure-6: Spermine inhibition of cyanine binding to λ DNA.....	17

TABLE

Table-1: Reversibility of DNA-model drug interactions by excess cyanine	16
---	----

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-complexation 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 binding⁹ 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 λ DNA 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%; $M_r = 202.3$) 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°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 10 μ M 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 (V_t) of 20 μ L 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 $\sim 25^\circ\text{C}$ using freshly diluted reagents in buffer. After 10minutes incubation, the reactions were diluted to 100 μ L V_t 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 (Optiplat, 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 \pm 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 (K_a) 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 4.0 μM respectively, when analyzed as $S - B$ and S/B at 425nm excitation wavelength (Fig. 1A). Likewise, the *preliminary* IC_{50} was 3.3 μM ($S - B$) 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 10 μM spermine regardless of the excitation wavelength (Figs. 1A and 1B).

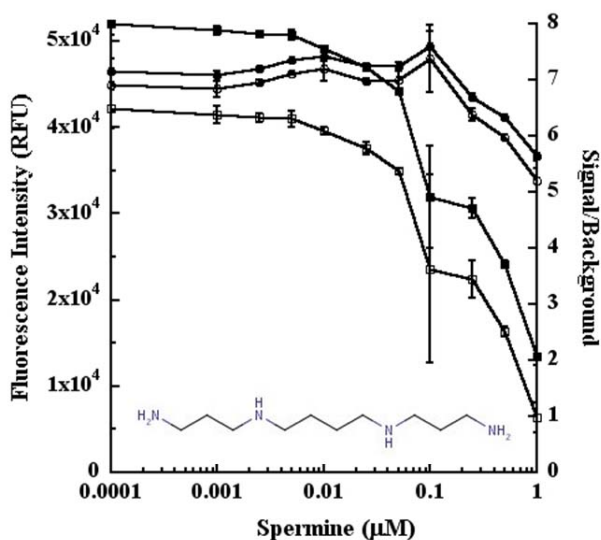


Fig. 1A: Spermine inhibition of cyanine binding to *Escherichia coli* genomic DNA using 425nm excitation wavelength. Increasing and indicated concentrations of spermine were mixed with 1.2fmol of DNA and 10 μM cyanine in 20 μL buffer. Fluorescence was measured at 470nm. The open symbols represent fluorescence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

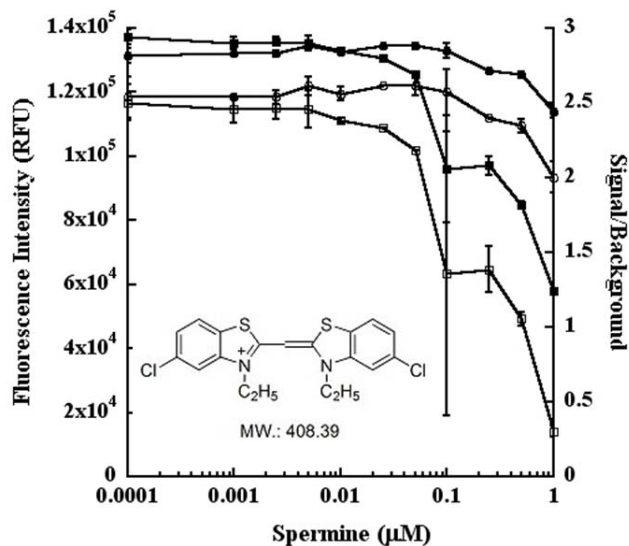


Fig. 1B: Spermine inhibition of cyanine binding to DNA using 450nm excitation wavelength. The reactions from Fig. 1A were interrogated using 450nm excitation wavelength. All other conditions are the same as those described for Fig. 1A. The inset shows the structure of cyanine.

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 20 μ L *Vt* in the absence of methanol, cyanine concentration was 10 μ M and spermine concentrations ranged from 0.1 to 100 μ M. When the well volume reached 100 μ L *Vt* due to incremental additions of methanol-water, cyanine concentration dropped to 2 μ M and spermine concentrations ranged from 0.02 to 20 μ M 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 100 μ L *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 (20 μ L *Vt*) to 32170 ± 3677 RFU in 16% (v/v) methanol concentration (100 μ L *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.

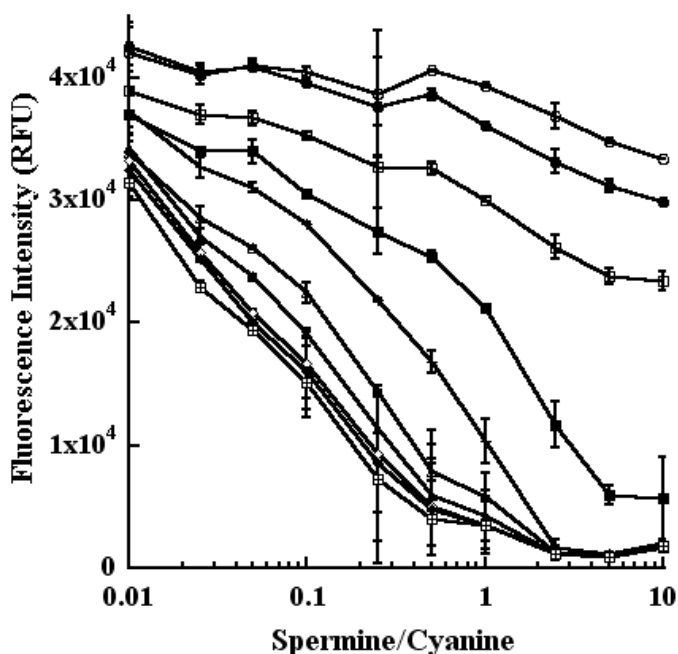


Fig. 2A: Effect of methanol on spermine inhibition of cyanine binding to *Escherichia coli* genomic DNA monitored by changes to the fluorescence intensity. Increasing concentrations of spermine were mixed with 1.3fmol of DNA and 10 μ M cyanine dye in 20 μ L *Vt*. The reaction wells were then diluted 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 (μ M): 10, 8, 6.7, 5, 4, 3.33, 2.85, 2.5, 2.22 and 2.0.

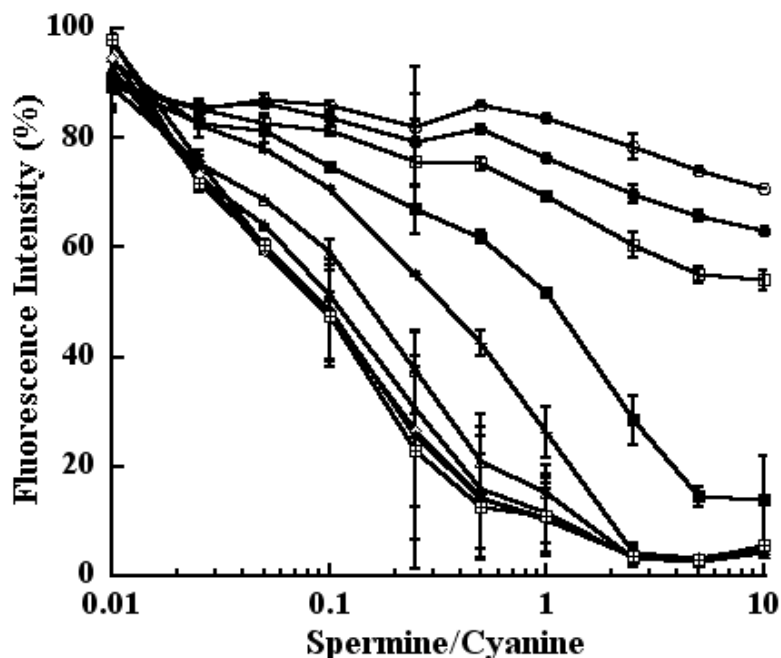


Fig. 2B: Effect of methanol on spermine inhibition of cyanine binding to DNA by changes to the normalized percent fluorescence emission. All conditions are as described for Fig. 2A except that the fluorescence intensity was transformed into percent values with emission in the absence of spermine being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

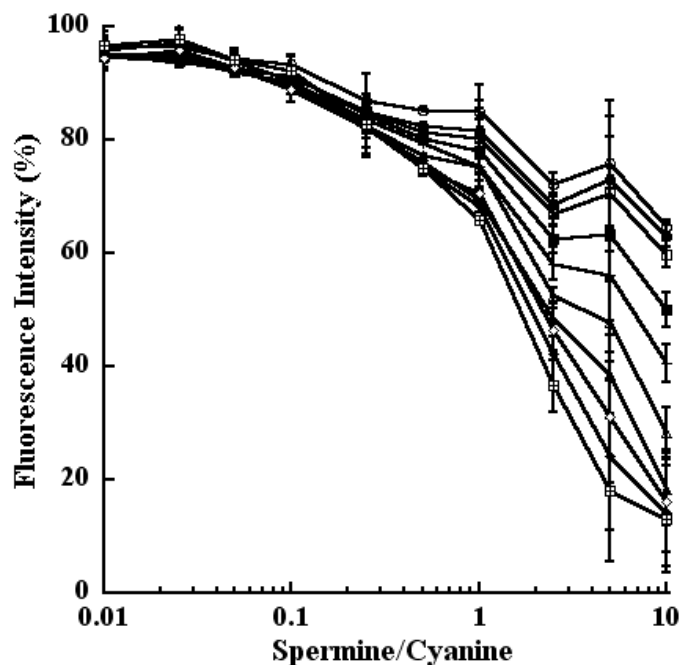


Fig. 3: Effect of buffer on spermine inhibition of cyanine binding to *Escherichia coli* genomic DNA. All conditions are *exactly* the same as described for Fig. 2A, except that incremental additions of buffer was used to dilute the binding reactions instead of methanol-water mixture. Consequently, the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

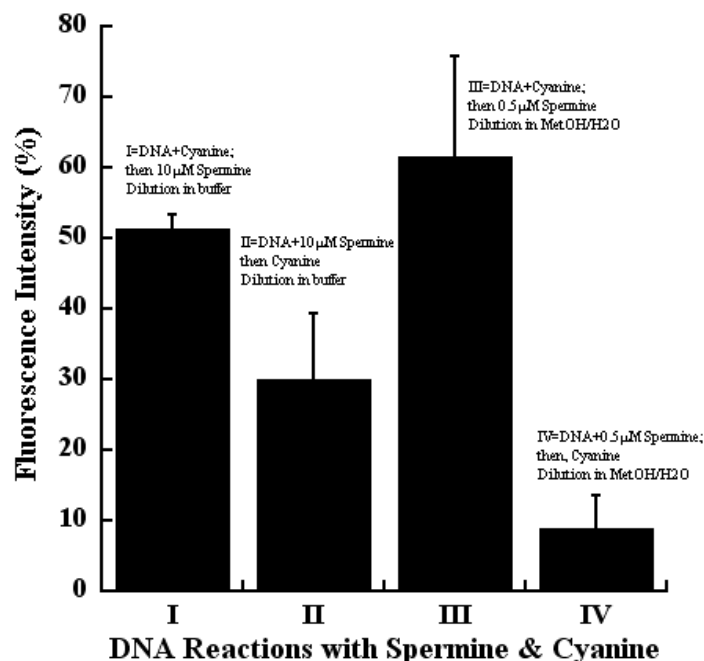


Fig. 4: Effect of reagents addition sequence upon spermine inhibition of cyanine binding to *Escherichia coli* genomic DNA. Binding reactions were carried out using 1.13fmol of DNA, 10 μ M cyanine and the indicated concentrations of spermine in 20 μ L of buffer. The DNA was mixed first with spermine or cyanine, incubated for 10 minutes followed by the addition of the other reagent (cyanine or spermine). The reaction volume was then diluted to 100 μ L 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 100 μ L *Vt*.

3.5 IC₅₀ and affinity constant

I next refined the spermine IC₅₀ for the DNA-cyanine J-aggregates (Fig. 5). The IC₅₀ was calculated as 0.35 \pm 0.03 μ M spermine. In the absence of methanol, when the binding was carried out in an equivalent volume of buffer, the IC₅₀ was 3.97 \pm 0.47 μ M 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 IC₅₀ values from experiments conducted more than 2 months apart. The association (binding) constant *Ka* in methanol was estimated as 2.86 x 10⁻⁶M. The *Ka* in buffer was 0.25 x 10⁻⁶M.

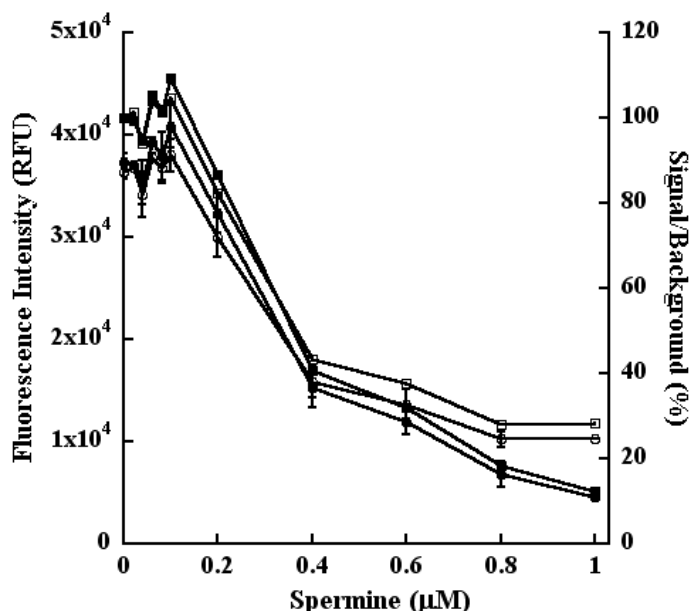


Fig. 5: IC₅₀ determination for spermine inhibition of cyanine binding to *Escherichia coli* genomic DNA. Indicated and increasing concentrations of spermine were added to 1.3fmol of DNA in buffer and allowed to incubate for 10 minutes as described in the legend to Fig. 4. Then 10µM cyanine (for 20µL *V_t*) 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) (20 μL V_t)*	% of Control Reactions (without spermine = 100%)		
	0.1 μM Spermine	0.25 μM Spermine	0.5 μM Spermine
20	59.3 \pm 4.0** (77.0 \pm 8.4)***	35.1 \pm 3.8 (38.2 \pm 6.3)	0 (0)
30	57.6 \pm 2.6 (80.5 \pm 3.3)	55.0 \pm 10.6 (75.5 \pm 8.3)	36.0 \pm 5.0 (100.0 \pm 7.4)
40	63.3 \pm 3.5 (99.6 \pm 5.4)	51.6 \pm 0.7 (85.7 \pm 0.0)	56.5 \pm 7.0 (94.6 \pm 9.7)

*20 μL V_t 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 100 μL with 80 μL 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 λ DNA (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 λ DNA-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 λ DNA was first exposed to spermine followed by cyanine. For example, with 25 μM cyanine, when λ DNA was bound to spermine first, the IC_{50} was 0.37 μM (Fig. 6). However, when the cyanine was *first* allowed to self-assemble upon λ DNA scaffold, the IC_{50} could not be calculated since the tracing became a flat line running parallel to the abscissa between 0.6 and 1.0 μM spermine. The IC_{50} for λ DNA at 10 μM cyanine was 0.17 – 0.25 μM spermine, slightly more potent than the values calculated for genomic DNA. However, the IC_{50} of 0.37 μM with 25 μM cyanine for λ DNA (Fig. 6) is close to the 0.35 μM calculated for *E. coli* genomic DNA obtained with 10 μM cyanine (*vide supra*).

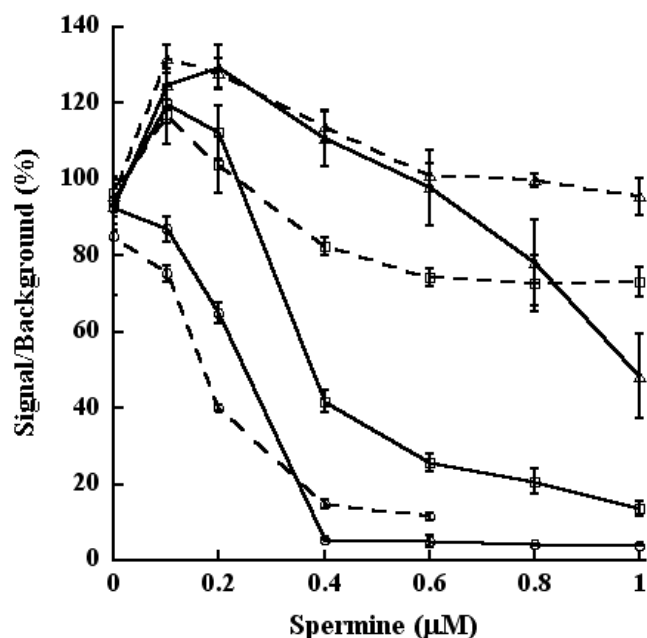


Fig. 6: Spermine inhibition of cyanine binding to λ DNA. Increasing and indicated concentrations of spermine was bound to 4fmol of DNA along with 10 μ M (circles), 25 μ M (squares) or 50 μ M (triangles) of cyanine. Solid tracings are binding reactions where the DNA was first mixed with spermine, incubated 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 λ DNA. For example, with prior exposure of λ DNA to spermine, at 10 μ M cyanine the IC_{50} was 0.25 μ M spermine (Fig. 6). In the presence of 25 μ M cyanine, the IC_{50} increased to 0.37 μ M and increased further to 0.97 μ M spermine when 50 μ M of self-assembled cyanine was displaced from λ DNA scaffold (Fig. 6). Between 25 μ M and 50 μ M 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 1.0 μ M 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 intramolecular reactions.⁹ Our studies with cyanine chemistries⁸ might also aid rational drug design⁴ 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 conformational 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 K_a 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 K_a is similar to $\mu\text{M } K_a$ (or K_d) 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 DNA³⁰ 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 similar²⁶ 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.”

6.0 REFERENCES

1. T. Kodadek, *Trends in Biochem. Sci.*, **1998**, 23, 79.
2. R. Palchadhuri and P. U. Hergenrother, *Curr. Op. Biotechnol.*, **2007**, 18, 497.
3. A. Erdem and M. Ozsoz, *Electroanal.*, **2002**, 14, 965.
4. I. Haq and J. Ladbury, *J. Mol. Recognit.*, **2000**, 13, 188.
5. K.E. Achyuthan and D.G. Whitten, *Comb. Chem. High Throughput Screen.*, **2007**, 10, 399.
6. W.C. Tse and D.L. Boger, *Acc. Chem. Res.*, **2004**, 37, 61.
7. Q. Huang and W-L. Fu, *Clin. Chem. Lab. Med.*, **2005**, 43, 841.
8. K.E. Achyuthan, J.L. McClain, Z. Zhou, D.G. Whitten and D.W. Branch, *Anal. Sci.*, **2009**, 25, 469.
9. S. Venkiteswaran, T. Thomas and T.J. Thomas, **2006**, 91, in: *Polyamine Cell Signaling: Physiology, Pharmacology and Cancer Research*, Humana Press, Inc., Totowa, NJ.
10. M. Wang, G.L. Silva and B.A. Armitage, 2000, *J. Am. Chem. Soc.*, **2000**, 122, 9977.
11. V. Vijayanathan, T. Thomas, A. Shirahata and T.J. Thomas, *Biochemistry*, **2001**, 40, 13644.
12. J. Szekely and K.S. Gates, *Chem. Res. Toxicol.*, **2006**, 19, 117.
13. H-F. Wang, R. Shen and N. Tang, *Eur. J. Med. Chem.*, **2009**, doi:10.1016/j.ejmech.2009.06.019.
14. R. Shen, P. Wang and N. Tang, *J. Fluoresc.*, **2009**, doi:10.1007/s10895-009-0507-6.
15. V.L. Singer, T.E. Lawlor and S. Yue, *Mutat. Res.*, **1999**, 439, 37.
16. B.P. Matselyukh, S.M. Yarmoluk, A.B. Matselyukh, V.B. Kovalska, I.O. Kocheshev, D.V. Kryvorotenko and S.S. Lukashov, *J. Biochem. Biophys. Meth.*, **2003**, 57, 35.
17. H. Tian and F. Meng, "Cyanine dyes for solar cells and optical data storage", ed. S-H. Kim, **2006**, Chap. 2, Elsevier, St. Louis, MO, USA, 47.
18. E.N. Timofeev, V.E. Kuznetsova, A.S. Zasedatelev and A.V. Chudinov, *Lett. Org. Chem.*, **2009**, 6, 71.
19. A. Furstenberg, T.G. Deligeorgiev, N.I. Gadjev, A.A. Vasilev and E. Vauthey, *Chem. Eur. J.*, **2007**, 13, 8600.
20. J. Pelta, F. Livolant and J-L. Sikorav, *J. Biol. Chem.*, **1996**, 271, 5656.
21. A.A. Ouameur and H-A. Tajmir-Riahi, *J. Biol. Chem.*, **2004**, 279, 42041.
22. B.C. Baguley, W.A. Denny, G.J. Atwell and B.F. Cain, *J. Med. Chem.*, **1981**, 24, 170.
23. A.J. Geall and I.S. Blagbrough, *J. Pharm. Biomed. Anal.*, **2000**, 22, 849.
24. A.J. Geall, D. Al-Hadithi and I.S. Blagbrough, *Bioconj. Chem.*, **2002**, 13, 481.
25. T. Biver, A. De Biasi, F. Secco, M. Venturini and S. Yarmoluk, *Biophys. J.*, **2005**, 89, 374.
26. D. Porschke, *Biochemistry*, **1984**, 23, 4821.
27. T. Antony, T. Thomas, A. Shirahata and T.J. Thomas, *Biochemistry*, **1999**, 38, 10775.
28. E. Rowatt and R.J.P. Williams, *J. Inorg. Biochem.*, **1992**, 46, 87.
29. T. Shangguan, D. Cabral-Lilly, U. Purandare, N. Godin, P. Ahl, A. Janoff and P. Meers, *Gene Therapy*, **2000**, 7, 769.
30. V.A. Bloomfield, *Biopoly.*, **1997**, 44, 269.

11. Distribution

#	Mail Stop	Name	Org #
1	MS-0899	Technical Library	9536 (electronic copy)
1	MS-0123	Donna Chavez, LDRD Office	1011 (electronic copy)
1	MS-1425	Komandoor Achyuthan	1714 (electronic copy)
1	MS-1425	Stephen Casalnuovo	1714 (electronic copy)
1	MS-1078	Wahid Hermina	1710 (electronic copy)

