Microarray Detection of Duplex and Triplex DNA Binders with DNA-Modified Gold Nanoparticles

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Abstract

We have designed a chip-based assay, using microarray technology, for determining the relative binding affinities of duplex and triplex DNA binders. This assay combines the high discrimination capabilities afforded by DNA-modified Au nanoparticles with the high-throughput capabilities of DNA microarrays. The detection and screening of duplex DNA binders are important because these molecules, in many cases, are potential anticancer agents as well as toxins. Triplex DNA binders are also promising drug candidates. These molecules, in conjunction with triplex forming oligonucleotides, could potentially be used to achieve control of gene expression by interfering with transcription factors that bind to DNA. Therefore, the ability to screen for these molecules in a high-throughput fashion could dramatically improve the drug screening process. The assay reported here provides excellent discrimination between strong, intermediate, and weak duplex and triplex DNA binders in a high-throughput fashion.

Keywords

DNA binding molecules; colorimetric screening; high-throughput; DNA-modified gold nanoparticles

JUSTIFICATION

Small molecules that can bind to duplex and triplex DNA are important for a variety of reasons. Typically, the association of either duplex or triplex DNA with a binding molecule results in a more stable complex relative to the duplex or triplex alone. When binding molecules are introduced into biological systems, they form DNA/small molecule complexes which exhibit increased stability compared to free DNA, and can induce cell death. The toxicity is a direct result of the small molecules interfering with the cells ability to interact with the DNA. As a consequence, small molecules with DNA binding properties are also potential anti-cancer drugs and gene regulation agents. Therefore, the ability to screen for duplex and triplex DNA binding molecules is an important step in the drug discovery process. In this manuscript, we present a high-throughput assay which is designed to screen for DNA binding molecules. The design of this assay allows for duplex and triplex binders to be easily distinguished. Importantly, this assay combines the unique properties of DNA-modified Au nanoparticles, which provide excellent discrimination between weak, intermediate, and strong DNA binders, with the high-throughput capabilities of microarray technology. This method provides benefits

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over fluorescence-based methods because it does not suffer from signal interference and provides improved discrimination between binders of different strengths.

INTRODUCTION

Since the initial development of Au nanoparticle probes through the covalent attachment of thiol-capped oligonucleotides to citrate stabilized gold nanoparticles in 1996[1], these composite nanomaterials have become the central component in several PCR-less DNA[2–9] and protein detection assays[10–12]. These assays benefit from the unique properties of these materials which are not displayed by either free DNA, Au nanoparticles without DNA, or Au nanoparticles that have been modified with oligonucleotides using different coupling schemes that lead to less efficient oligonucleotide loading. These properties include: 1) enhanced binding properties which directly lead to increased sensitivity[13] and 2) sharp DNA melting transitions which dramatically increase the level of selectivity of DNA binding[2]. In addition, the intense optical properties of the Au nanoparticles provide a strong signal by which to monitor the melting behavior of DNA, which has a relatively weaker spectroscopic signature. The combination of these properties has led to assays with impressive analytical capabilities with respect to sensitivity, selectivity, and assay readout for both nucleic acid and protein analytes.

Recently, DNA-modified Au nanoparticles have been used for the colorimetric detection of duplex[14] and triplex[15] DNA binding molecules.[16, 17] Researchers are interested in probing the interactions between small molecules and DNA in order to obtain leads for new anti-cancer and gene regulation agents. Many duplex DNA binders such as doxorubicine, daunorubicin and amsacrine have been identified and used as anticancer drugs.[18] They are known to reversibly interact with duplex DNA to form a drug/DNA complex, and by doing so disrupt cellular replication. Triple helix specific DNA binding molecules, or triplex binders, are also promising drug candidates. These molecules, in conjunction with triplex forming oligonucleotides, could potentially be used to achieve control of gene expression by interfering with transcription factors that bind to DNA.[19–21]

The recently developed DNA-modified Au nanoparticle-based colorimetric screening methods display excellent discrimination between weak, intermediate and strong duplex and triplex DNA binders[16, 17]. However, with the development of combinatorial chemistry where many compounds can be synthesized within a short time period,[22, 23] high-throughput screening techniques are necessary to screen large libraries for potential drug candidates.[24–26] In the past, typical screening processes have included UV-vis melting experiments, competitive dialysis, and mass spectroscopy.[21, 27–29] Unfortunately, these methods are not applicable to high-throughput screening. Only recently have protocols been developed which are compatible with high-throughput screening techniques.[16, 17, 30, 31] Herein, we present a new high-throughput technique, based on microarray technology, which incorporates the high discrimination capabilities of DNA-modified Au nanoparticles for the screening of duplex and triplex DNA binding molecules.

These assays are based on a perturbation of the DNA melting temperature (T_m) induced by the presence of DNA binders. Strong DNA binders result in a greater increase in Tm whereas weaker DNA binders have very little effect on the Tm. Since DNA functionalized Au nanoparticles have such an intense optical signature, one can work at higher ratios of potential binder:probe DNA, which leads to larger absolute changes in T_m as compared with conventional UV-vis approaches. This increased perturbation expands the temperature window that can be used to screen for duplex and triplex DNA binders. Generally, the strength of binding between the drug and target molecule correlates with the drug's biological activity and, therefore, it is important to obtain a quantitative measure of the difference in binding

capabilities of the molecules that make up a particular library.[32] The authors note that there are a few cases where strong DNA binding molecules do not increase the T_m associated with the bound DNA.[33] However, this is an uncommon characteristic, and the high-throughput screening assay described here is directed towards screening the more general DNA binders which make up the majority of these molecules and not special cases.

The assay described herein relies on scanometric detection and the use of a commercial reader, the Verigene ID.[4] In a typical experiment, synthetic alkyl amine functionalized oligonucleotides are arrayed on a glass chip via literature procedures (see Methods). Gold nanoparticles, functionalized with a complementary DNA sequence, are hybridized to the chip. A sample holder with 10 different wells that reside over the microarrays is attached to the microscope slide with the arrays. Each well is filled with an aqueous solution containing a different potential DNA binder. After hybridization, the probes are developed with a silver enhancement solution, which increases the size and light scattering of the Au nanoparticle probes, Scheme 1 and 2. The amount of light scattering from each spot within the microarrays is measured using a Verigene ID scanner. To elucidate strong, intermediate and weak DNA binders, the incubation process with the DNA binder is performed under more stringent conditions by increasing the temperature or decreasing the salt concentration of the solution. As conditions become more stringent, the DNA interconnects require DNA binders to increase the stability of the linking DNA in order for the Au nanoparticles to remain intact on the chip surface. The dehybridization of the Au nanoparticles on the chip surface directly correlates with a reduction of signal post silver enhancement, thereby allowing one to differentiate DNA binders of different strengths. To demonstrate this technique, we chose seven known duplex DNA binders (4′,6-diamidino-2-phenylindole (DAPI), ellipticine (EIPT), amsacrine (AMSA), daunorubicin (DNR), anthraquinone-2-carboxylic acid (AQ2A), ethidium bromide (EB), and 9-aminoacridine (9-AA)). The chosen duplex binders represent a range of binding strengths and include both groove binders (DAPI, DNR) and intercalators (EIPT, AMSA, AQ2A, EB, and 9-AA). Since only a few triplex binders are known, our choices of triplex binders were limited and include coralyne (CORA), a weak binder, and benzo[e]pyridoindole (BePI), a strong binder.

EXPERIMENTAL SECTION

All oligonucleotides used in this paper were purchased from Integrated DNA Technologies. Ellipticine was purchased from Biomol (Plymoth Meeting, PA). Coralyne was purchased from Acros Organics (Geel, Belgium). All other chemicals and buffer solutions were prepared using reagents purchased from Sigma-Alrdich (St. Louis, MO).

DNA Modification of Gold Nanoparticles

Au nanoparticles (13 nm diameter), prepared by citrate reduction of $HAuCl_4$ were used to prepare all probes.[34] Gold nanoparticles were functionalized by derivatizing aqueous Au colloid with 3′-alkylthiol-modified oligonucleotides (final concentration of oligonucleotides ~4 μM). After 12 hours, the phosphate concentration was increased from 0 to 10 mM by adding 1.0 M pH 7 phosphate buffer (NaH_2PO_4/Na_2HPO_4). This was followed by a series of salt additions in order to slowly increase the loading of the oligonucleotides. Initially, 2 M NaCl was added dropwise to increase the salt concentration to 0.05 M NaCl. The solution was allowed to stand for 6–8 h. This process was repeated to increase the salt concentration to 0.1, 0.2 and 0.3 M NaCl. To remove excess thiol-DNA, the solution was centrifuged using 1.5 mL Eppendorf tubes (Fisher Scientific) (30 min at 15 000 rpm). Following removal of the supernatant, the precipitate was suspended in NANOpure® water. This process was repeated five times with a final resuspension in 0.5 M NaCl buffer (10 mM PBS, 0.001 % NaN_3).

Determination of Melting Temperature of Control DNA (No Nanoparticles)

To determine the T_m of the duplex DNA in the presence of DNA binders, duplex DNA and a DNA binder were combined in 0.3 M NaCl 10 mM PBS to final concentrations of 2 μM and 5 μM, respectively. To determine the T_m of the triplex DNA in the presence of DNA binders, triplex DNA (1:1:1 each DNA strand) and a DNA binder were combined in 0.5 M NaCl 10 mM PBS to final concentrations of 1 μM and 6 μM, respectively. UV-vis melting curves were measured on a CARY 500 spectrophotometer equipped with a circulating bath. The melting process was monitored by measuring the change in extinction at 260 nm. The solutions were stirred continuously with a magnetic stir bar as the solution temperature was increased at a rate of 0.5 °C/min. The first derivative generated from the melting curve was used to determine the T_m.

Preparation of DNA Chip Array

N-Hydroxysuccinimide-activated Codelink glass microarray slides (Amersham, G. E. Healthcare) were spotted with alkylamine-modified oligonucleotides (Duplex Array:5′-TAA CAA TAA-A10-NH$_2$-3′, Triplex Array: 5′-TC TTT TTT CTT CTT AAC TCG-PEG-NH$_2$-3′, Integrated DNA Technologies, Inc.) according to the slide manufacturer's protocol. The oligonucleotides were printed in triplicate using a GME 418 robotic pin-and-ring microarrayer (Affymetrix).

Scanometric Detection of Duplex and Triplex DNA Binding Molecules

Glass slides containing the microarrays were fitted with sample chambers (Nanosphere, Inc.), creating 10 individually addressable wells. Gold nanoparticles were added to each well (1nM) in 10 mM PBS with varying concentrations of NaCl (0.1–0.3 M). For detection of triplex binders, 150 nM DNA-3 was added. DNA binding molecules (5 μM) were added to the sample wells. Duplex DNA binders included 4′,6-diamidino-2-phenylindole (DAPI), ellipticine (EIPT), amsacrine (AMSA), daunorubicin (DNR), anthraquinone-2-carboxylic acid (AQ2A), ethidium bromide (EB), and 9-aminoacridine (9-AA). Triplex DNA binders were coralyne (CORA) and benzo[e]pyridoindole (BePI). Incubation with the DNA binders was allowed to proceed for 30 min for duplex DNA binders at a specified temperature and 1 hour for triplex DNA binders at 25 °C. After hybridization, the slides were washed three times with 0.5 M NaNO$_3$ containing 0.02% Tween 20. The microarrays were dried using a benchtop spinner. Gold probes were developed with silver enhancement solution (Ag$^+$/Hydroquinone, Nanosphere, Inc.) (1 mL/chip, 1.5-min development time for duplex DNA binders and 3 min for triplex binders). The Au nanoparticles catalyze the reduction of silver, resulting in silver deposition around the nanoparticles. The reaction was terminated by washing the arrays with NANOpure® water followed by spin-drying. The light scattering from the silver enhancement is then measured by a high-resolution Verigene ID scanner (Nanosphere, Inc.).

RESULTS

Scanometric detection of duplex DNA binders was performed at different temperatures, Figure 1. At 25 °C, all seven binders and the control displayed signal after silver development. Performing the assay at 30 °C resulted in signal from only arrays incubated with DAPI (strong binder), DNR and EB (intermediate binders). Increasing the assay temperature further to 35 °C resulted in signal from only DAPI, a strong duplex binder. Scanometric detection of triplex DNA binders was performed at varying salt concentrations, Figure 2. At 0.3 M NaCl, signal was obtained from both triplex binders, BePI and CORA, while no discernable signal was obtained from the duplex DNA binders. Reducing the salt concentration to 0.2 and 0.1 M NaCl resulted in signal from only BePI, a strong triplex binder.

Control experiments with free DNA (no nanoparticles) were performed to verify the trend of DNA binding strength found using the scanometric method. Melting temperatures are shown for the duplex and triplex binders in Table 1 and Table 2, respectively.

DISCUSSION

Detection of Duplex DNA Binders

Capture strands of alkylamine-modified oligonucleotides were spotted on a glass chip using a microarrayer. The microarray was then exposed to DNA-modified Au nanoparticles (complementary to the capture sequence) followed by addition of a duplex DNA binder. The nanoparticle-bound DNA hybridizes to the capture strand and forms a duplex, simultaneously immobilizing the nanoparticle on the array and providing a binding site for the duplex DNA binder, Scheme 1. The array was washed with buffer and the gold probes are developed with silver enhancement solution and the signal is measured with a Verigene ID.

The hybridization of complementary DNA is a reversible process and the stability of the resulting duplex is affected by several factors such as temperature and salt concentration. In addition, the presence of duplex DNA binders increases the stability of the DNA duplex and is reflected by an increase in the duplex T_m. The strength of binding of the duplex DNA binder is represented by the magnitude of increased T_m.[32] Therefore, by performing the nanoparticle hybridization process in the presence of different duplex DNA binders as a function of temperature, the relative binding strength of duplex DNA binders can be determined, Figure 1.

Analysis of seven known duplex DNA binders was performed to demonstrate the ability of this method to discriminate between strong, intermediate and weak binders. At 25 °C, all seven duplex DNA binders and a control showed positive signal after silver development. Increasing the temperature to 30 °C during the hybridization process eliminates signal from the control and the weak duplex DNA binders, leaving the intermediate (EB and DNR) and strong (DAPI) DNA binders. Further increasing the temperature eliminates the signal from the intermediate strength binders, leaving only the strong duplex DNA binder, DAPI.

These results correlate well with control experiments which measure the T_m of free solution-phase DNA in the presence of the seven duplex DNA binders, Table 1. The relative trend in binding strength determined from the control experiments is DAPI > DNR > EB > other intercalators. This is in agreement with the trend in relative binding strengths determined by the chip-based scanometric method.

Detection of Triplex DNA Binders

Capture strands of alkylamine-modified oligonucleotides are patterned on a glass chip using a microarrayer. The microarray is then exposed to a solution containing three components: 1) complementary DNA (no nanoparticles), 2) DNA functionalized Au nanoparticles (DNA is non-complementary to the arrayed DNA) and 3) DNA binders, Scheme 2. The complementary solution-phase DNA, called DNA-3 in Scheme 2, hybridizes to the microarray and forms a duplex. The Au nanoparticle-bound DNA has the correct sequence to form a triplex with the surface bound duplex through sequence-specific Hoogsteen base pairing.[35] The triplex structure is relatively unstable at room temperature and will only form in the presence of triplex DNA binding molecules. The presence of the triplex DNA binding molecules increases the stability of the triplex, immobilizing the Au nanoparticles onto the microarray. The array was then washed with buffer and the gold probes were developed with silver enhancement solution. The light scattering from the microarray was measured using a Verigene ID scanner.

The assay was performed in the presence of two triplex binders, seven duplex DNA binders, and a control (no DNA-3). Similar to the DNA duplex, the formation of the DNA triplex is a reversible process which is affected by both temperature and salt concentration. However, compared to the duplex, the stability of the triplex is much more sensitive to salt concentration. [36] Therefore, to determine the relative binding strength of the triplex DNA binders, the assay was performed at room temperature at varying salt concentrations, Figure 2. At 0.3 M NaCl, both the strong (BePI) and weaker (CORA) triplex binders show signal. At decreased salt concentrations (0.2 and 0.1 M NaCl), the stability of the triplex is decreased and signal is only seen in the presence of the stronger triplex binder (BePI). No signal is seen in the presence of the duplex DNA binders indicating that the assay is specific for identifying triplex DNA binders.

These results correlate well with control experiments which measure the T_m of free solution-phase DNA in the presence of the two triplex DNA binders and the seven duplex DNA binders, Table 2. A melting transition associated with the triple helix was found only for experiments performed in the presence of BePI (34.8 °C) and CORA (17.1 °C) where BePI is determined to be the stronger binder. This is in agreement with the results determined by the scanometric method described above.

In conclusion, we have designed a microarray assay for screening the relative binding discrimination capabilities afforded by the DNA modified Au nanoparticles with the affinities of duplex and triplex DNA binders. This assay combines the high high-throughput capabilities of DNA microarrays. The detection and screening of duplex DNA binders are important because these molecules, in many cases, are potential anticancer agents as well as toxins. Triplex DNA binders are also promising drug candidates. These molecules, in conjunction with triplex forming oligonucleotides, could potentially be used to achieve control of gene expression by interfering with transcription factors that bind to DNA. Therefore, the ability to screen for these molecules in a high-throughput fashion could dramatically improve the drug screening process.

Acknowledgements

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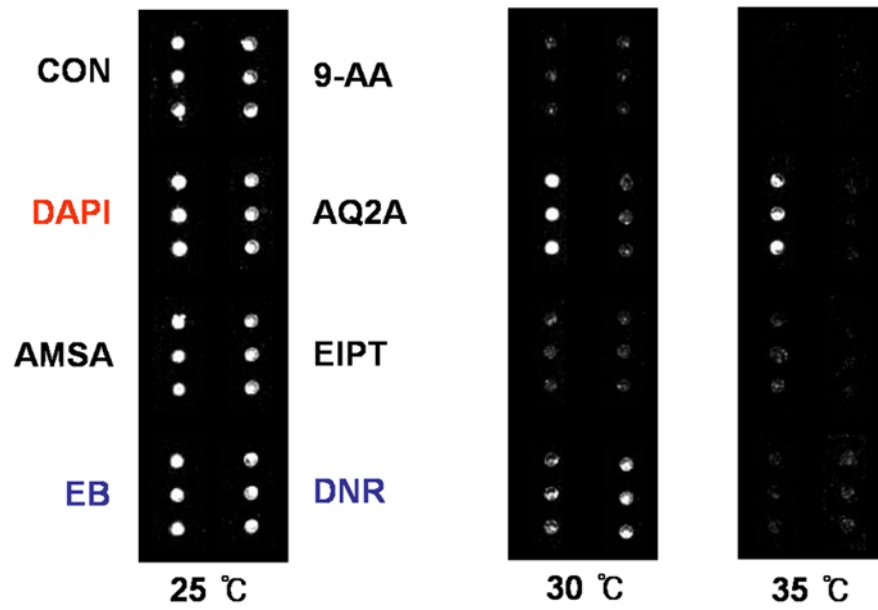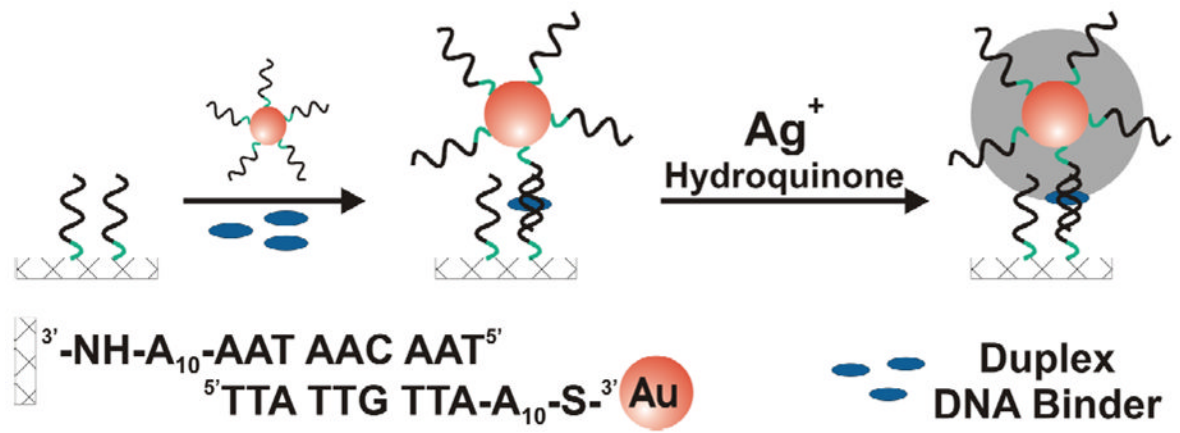
Figure 1.
Light scattering image of a microarray post silver development. The spots are scattered light signifying duplex DNA binding molecules.
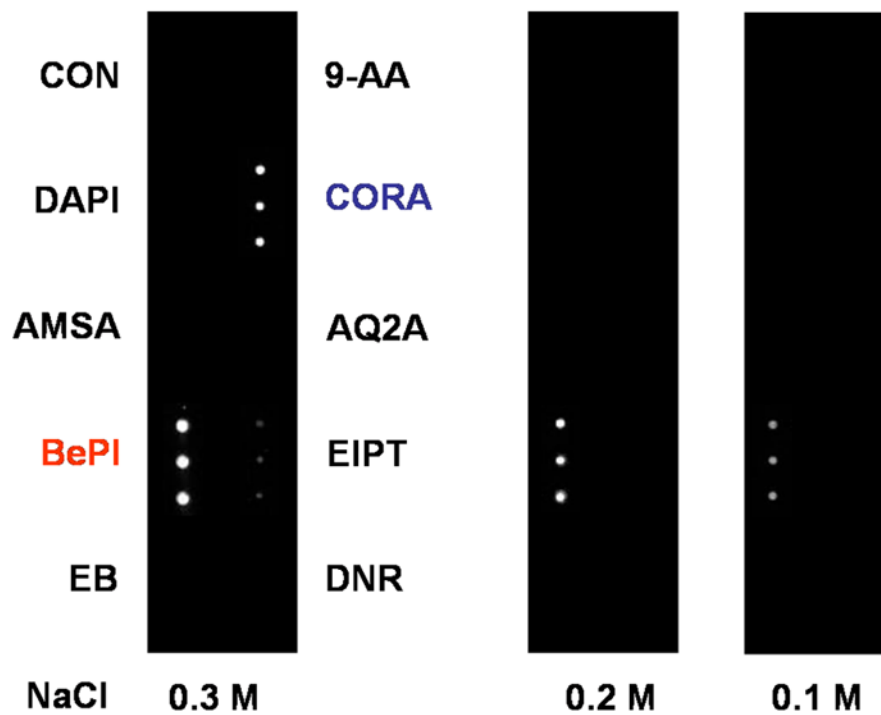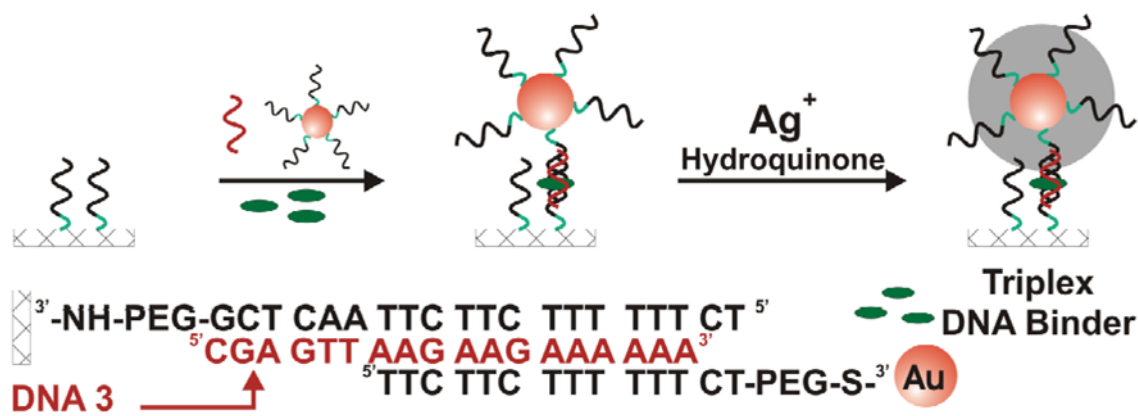
Figure 2.
Light scattering image of a microarray post silver development. The spots are scattered light signifying triplex DNA binding molecules. Experiments were performed at 25 °C.

Scheme 1.
scanometric detection of duplex DNA binders on a microarray.

Scheme 2.
Scanometric detection of triplex DNA binders on a microarray.

Table 1

Melting temperatures of solution-phase unmodified DNA in the presence of duplex DNA binders: 4′,6-diamidino-2-phenylindole (DAPI), ellipticine (EIPT), Amsacrine (AMSA), daunorubicin (DNR), anthraquinone-2-carboxylic acid (AQ2A), ethidium bromide (EB), and 9-aminoacridine (9-AA)).

Duplex Binders	T_m (°C)
Control (No DNA Binder)	19.1
DAPI	27.8
DNR	25.4
EB	21.8
EIPT	20.5
AMSA	19.5
AQ2A	19.4
9-AA	19.3

Table 2

Melting temperatures of solution-phase unmodified DNA in the presence of triplex DNA binders: coralyne (CORA) and benzo[e]pyridoindole (BePI) and duplex DNA binders: 4′,6-diamidino-2-phenylindole (DAPI), ellipticine (EIPT), Amsacrine (AMSA), daunorubicin (DNR), anthraquinone-2-carboxylic acid (AQ2A), ethidium bromide (EB), and 9-aminoacridine (9-AA). T_m3-2 indicates a melting transition from a triplex to a duplex. T_m2-1 indicates a melting transition from a duplex to single stranded DNA.

Compounds	T_m3-2 (°C)	T_m 2-1 (°C)
Control	ND	58.9
9-AA	ND	59.1
AQ2A	ND	59.1
AMSA	ND	59.2
EIPT	ND	59.3
EB	ND	59.8
CORA	**17.1**	60.1
BePI	**34.8**	61.4
DNR	ND	61.7
DAPI	ND	66.3