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LETTER

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Does it bind? An instant binding assay for DNA oligonucleotide interactive small molecules

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Ultrafiltration analysis has been used to screen DNA oligonucleotide interactive small molecules for the first time, providing an almost instantaneous indication of the binding affinity, and was used to identify a new class of DNA interactive molecules based on a range of dihydro-imidazo-phenanthridinium-based framework; this methodology provides a straightforward yes/no answer to small molecule DNA binding.

The design and discovery of molecules that can interact with DNA represents a tremendously important area of science. The scientific community has long realized the potential of such molecules in the fields of genomics[1] and drug design/delivery,[2] with more recent uses as DNA probes[3] and dyes.[4] Thus, the rapid growth of this area of science has required improvements in DNA screening techniques, with an increasing desire for systems that allow rapid results through minimal input. The long-term objective is to develop a reliable system that instantly provides a yes/no answer to potential small molecule DNA binders.

Presently the techniques used to measure DNA binding include isothermal titration calorimetry (ITC),[5] ethidium displacement[6,7] and fluorescence titration experiments.[8] However these techniques require access to relatively specialist equipment, which is often expensive, and have long experimental times. A more recently developed method, generally used for investigation of ligand–protein interaction, is competition dialysis.[9–12] This method uses a semi-permeable membrane to separate the free ligands from a mixture of free ligand and bound protein. Although this technique is effective due to its simplicity and cost effectiveness it involves long experimental times, as equilibrium must be reached within the system. The requirement for precision in the difference of ligand concentrations between the two compartments is also problematic when studying low affinity binding. Improvements in this technique have led to ultrafiltration,[13] where the free and bound ligand are separated by filtration under pressure through a permselective membrane, allowing for rapid investigation of large numbers of samples. Until now, ultrafiltration has been used to study the interactions between proteins and different ligands.[13,14] Difficulties arise when charged solutes are used, as additional repulsive electrostatic interactions between the membrane and solute must be considered.[15]

We wanted to see if we could use ultrafiltration as a rapid way to study a new class of potentially DNA interactive small molecules, developed recently by us, involving a one-pot, three-step reaction between primary amines and 2-bromoethyl phenanthridinium bromide. This leads to dihydro-imidazo-phenanthridinium compounds (DIP),[16] which contain a large, planar, polyaromatic core and so are likely to have a high affinity for DNA, probably *via* intercalating between two adjacent base pairs in the duplex.[17] The simplicity of this reaction allows a large library of compounds to be synthesized in a short time. For this reason we require an instant binding answer for these molecules. The technique we present in this paper, although it does not provide an accurate binding affinity, does provide an immediate yes/no answer to DNA binding. This could be extremely important, *e.g.* for synthetic chemists who wish to screen an initial library, before embarking on more specialized and time consuming ITC measurements to study molecules with affinity for DNA in more detail.

Herein we report the development of this method for use in DNA oligonucleotide binding studies, by the introduction of centrifugal force, allowing the fast and efficient screening of small molecules for possible binding affinity with DNA oligonucleotides. Compounds containing both aromatic and aliphatic side chains, with various degrees of hydrophobicity, were chosen to demonstrate the versatility of the methodology along with the variation in relative binding affinities associated with changes in structure, see Table 1.

The eight different DIP-based molecules and ethidium bromide (EtBr) have been investigated with respect to DNA oligonucleotide binding using our ultrafiltration analysis technique (see experimental for full details). Each experiment was repeated three times and all the results obtained within 90 minutes, using a simple centrifuge and UV spectrometer. The binding profiles for the molecules examined using this technique are shown in Fig. 1. EtBr shows the highest percentage binding affinity for the DNA oligonucleotide (94%) and molecule **1**, containing a different aromatic ring system from the DIP-based molecules, the lowest affinity for the DNA oligonucleotide (13%). This molecule is synthesized from a quinazoline derivative, containing one less aromatic ring than the phenanthridine derivatives. Of the DIP compounds, molecule **4** has the greatest binding (45%).

Validation of the results was obtained by ITC (Table 2). A series of 29 portions of DIP solution (10 μL; 1 mM) were injected into the DNA oligonucleotide (1.7 mL) and the resulting change in heat for each injection measured. This value is plotted against the molar ratio of the ligand added affording an isotherm that could be fitted using standard techniques,[18] yielding the binding constant for the association. The same type and concentration of DNA oligonucleotide was used.

Molecule **4** has the greatest K_a and percentage binding with DNA. This can be explained by the presence of the glycol

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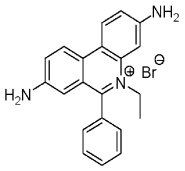
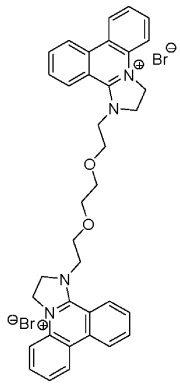
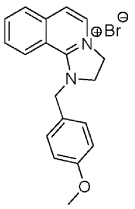
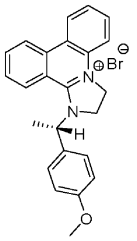
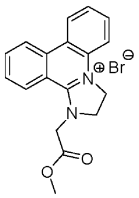
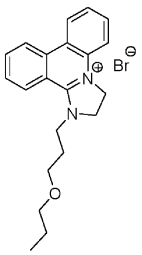
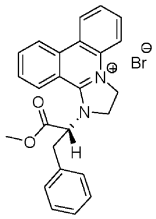
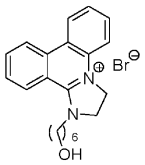
Table 1 Compounds examined using this methodology

Compound	Structure	Compound	Structure
EtBr		**4**	
1		**5**	
2		**6**	
3		**7**	

linking chain, which allows flexibility within the molecule.[19] Molecule **4** is also capable of bis-intercalation due to the presence of two aromatic regions, allowing for both inter- and intrastrand cross-linking. The linking unit may also allow association within the minor groove due to hydrophobic effects.[20] Hydrophobic effects may also account for the difference between isomer DIPs **7** ($K_a = 2.25 \times 10^4$, % binding = 36.1) and **6** ($K_a = 9.9 \times 10^3$, % binding = 23.5), whereby the shorter distance between the aromatic core and the ether oxygen of molecule **6** decreases its hydrophobicity relative to

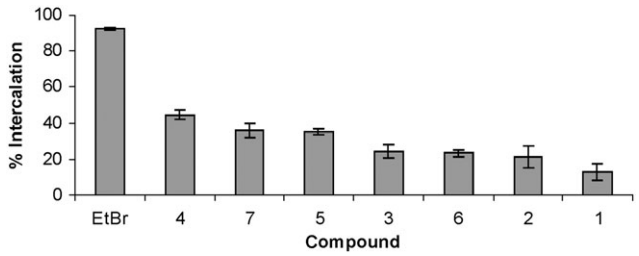


Fig. 1 Binding profiles of different DNA oligonucleotide binders as determined by the centrifugation method.

Table 2 DNA binding results obtained from ITC correlated with the percentage binding to DNA found using the centrifugation technique

Compound	K_a (ITC) (M^{-1})	% Binding
EtBr	$7.1 \times 10^4 \pm 3942$	92.3 ± 0.7
4	*ca.* 7.00×10^4	44.6 ± 2.4
7	$2.25 \times 10^4 \pm 1649$	36.1 ± 4.0
5	$1.16 \times 10^4 \pm 1880$	35.0 ± 1.3
3	$0.763 \times 10^4 \pm 23.8$	24.4 ± 3.8
6	$9.9 \times 10^3 \pm 1736$	23.5 ± 1.8
2	$3 \times 10^3 \pm 1734$	21.3 ± 5.9
1	No binding	13.0 ± 4.9

7. This trend in K_a values corroborated the results determined from the centrifugation technique. Although the DIP molecules synthesized show a marginally lower affinity for DNA than EtBr, their ease of synthesis and amenability to systematic structural variation, through a one-pot, three-step reaction system, compared to that of EtBr make them very interesting systems for further development.

To conclude, a straightforward, efficient method of screening DNA oligonucleotide binding has been developed, utilizing centrifugal force and ultrafiltration. The simplicity of the method enables initial DNA binding studies for small molecules to be carried out effortlessly in the lab, producing instant yes/no results, rather than a detailed and highly accurate set of data (however it should be noted that the ITC *does corroborate* with the results gained from our method). This enables synthetic chemists to screen their own molecular candidates extremely quickly in cases where a yes/no indication would be useful to refine their molecular design strategy to improve binding *etc*. This technique could therefore be used before more detailed studies that themselves allow, for instance, the deduction of accurate binding constants and sequence specificity. Furthermore we have successfully applied this methodology to the discovery of a new class of DNA interactive molecules based on dihydro-imidazo-phenanthridinium, and the results have been confirmed by ITC measurements.

Experimental

The ultrafiltration analysis experiment was carried out using a known intercalator, ethidium bromide (EtBr), and DIP conjugates, the structures of which are shown in Table 1.

Solutions of each of the compounds were prepared (100 μM) with PIPES buffer and 0.18 mL pipetted into separate Microcon® YM-3 sinters. To these sinters 0.32 mL of Dickerson dodecamer DNA (0.48 mM; $M = 3640$) in PIPES buffer (pH 7) were added. The size of sinters used depends solely on the molecular weight of the DNA used, as it must be unable to pass through the sinter's pores. The sinters are then centrifuged (12 100 $\times g$) for 60 min. After this time, around 50 μL of the solution remains on the top of the sinter, containing DIP molecules bound to DNA as they are too large to pass through the sinter's pores. Any unbound DIP molecules will be pulled through the pores. The volumes of material remaining on the top and bottom of each of the filters are collected, recorded and their UV absorbance at 360 nm measured. The material was diluted with PIPES (0.45 mL). The percentage of intercalation was calculated using $(A_t \cdot V_t/[(A_t \cdot V_t) + (A_b \cdot V_b)]) \cdot 100$, where A_t and A_b are the absorbances of the materials in the top and the bottom of the sinters and V_t and V_b are the respective volumes. Results are expressed as mean ± standard deviations ($n = 3$) (Fig. 1). No UV absorbance was observed at 360 nm for Dickerson dodecamer DNA. A control containing only Dickerson dodecamer DNA (0.48 mM; 0.5 mL) was simultaneously set up. No UV absorbance was measured in the top or bottom of the sinter.

A further control containing only the DIP molecules (100 μM; 0.5 mL) was set up without any detection of

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absorbance in the top of the sinter (original concentration was detected on the bottom of the sinter).

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