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Use of capillary electrophoresis in the study of ligand–DNA interactions

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ABSTRACT

Free solution capillary electrophoresis (FSCE) has been used to separate two non-self-complementary 12mer oligonucleotide duplexes: d(AAATTATATTAT)·d(ATAA-TATAATTT) and d(GGGCCGCGCCGC)·d(GCGGCGCG-GCCC). Titration of mixtures of the two oligonucleotides with model intercalators (ethidium bromide and actinomycin D) and minor groove binders (netropsin, Hoechst 33258 and distamycin) has shown the suitability of FSCE as a method to study the sequence selectivity of DNA binding agents. Binding data have shown cooperativity of binding for netropsin and Hoechst 33258 and have provided ligand:DNA binding ratios for all five compounds. Cooperativity of netropsin binding to a 12mer with two potential sites has been demonstrated for the first time. Ligands binding in the minor groove caused changes in migration time and peak shape which were significantly different from those caused by intercalators.

INTRODUCTION

There is a wide range of established techniques for the study of ligand–DNA binding, from the simple measurement of UV absorption and melting temperature to demanding but highly informative methods using NMR and X-ray crystallography (1–4). In general, separation techniques are not used, with the exception of footprinting, in which gel electrophoresis is used to separate DNA fragments of different lengths and to compare the patterns obtained after DNA cleavage in the presence and absence of a suspected binding species (5–6).

DNA footprinting has recently been improved to allow quantitative comparison of the affinity of a ligand for different base pair sequences. Quantification has demonstrated remarkable selectivity of binding, for example showing a preference for binding of netropsin, distamycin and Hoechst 33258 to AATT and AAAA rather than TTAA and TATA, in contrast to previous general findings of a preference for AT over GC sequences (7). Selectivity of binding is of the utmost importance in designing new ligands for the treatment of disease. Footprinting has been the best method for this purpose for a number of years, but suffers from some experimental disadvantages where large numbers of compounds have to be tested. For simple intercalators the sequence preference is more difficult to detect by standard footprinting methods, since they dissociate rapidly from the binding sites (8–10).

In the present paper we describe a capillary electrophoresis method which is fast, economical and highly informative. The development of such a method for the comparative study of DNA binding affinity, using a competition experiment, required that oligonucleotides of different base pair sequence be separable. While there have been reports of electrophoretic separations based on length, down to 1 bp resolution, using gel-filled capillaries (11), there were no reports of sequence-based separation other than those using affinity labelling (12), which was not suitable for our purpose. In preliminary studies we were surprised and gratified to find that the oligonucleotides $d(AT)_{12}$ and $d(GC)_{12}$ were separable on a simple silica capillary (13), using borate buffer at pH 8.0–8.5.

When we attempted to use $d(AT)_{12}$ and $d(GC)_{12}$ in a model experiment to test whether capillary electrophoresis could be used to detect selective ligand binding, the results were encouraging. Unfortunately, the electropherograms showed multiple peaks for the oligonucleotides in the presence of DNA binding ligands which we attributed to the propensity of these self-complementary oligomers to associate in different ways, i.e. hairpin loops as well as interstrand base pairing. On this basis we designed two directly analogous non-self-complementary AT and GC dodecamers, with a random element in the sequence. The two double-stranded oligomers d(AAATTATATTAT)·d(ATAATATAATTT) and d(GGG-CCGCGCCGC)·d(GCGGCGCGGCCC) were used for the ligand binding studies, initially in competition experiments and then singly when it became apparent that a large amount of useful information was obtainable.

MATERIALS AND METHODS

Materials

Boric acid (reagent grade), sodium tetraborate decahydrate (electrophoretic grade), actinomycin D (AcD), ethidium bromide (EtB), distamycin (distamycin A, Dst) and Hoechst 33258 (Ht) were purchased from Sigma Chemical Co. (St Louis, MO). Netropsin (Nt) was a gift from Dr A.R.Pitt (University of Strathclyde). The non-self-complementary oligonucleotides d(AAATTATATTAT)·d(ATAATATAATTT) (AT 12mer) and d(GGGCCGCGCCGC)·d(GCGGCGCGGCCC) (GC 12mer) were obtained as single strands from Cruachem Ltd (Glasgow, UK). Concentrations of the oligonucleotides were measured by

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UV spectrophotometry at 260 nm using the molar absorptivities provided by the supplier. Samples of the oligomers were diluted in distilled water (0.5–1 ml) and stored at –20°C until use. The single-stranded oligonucleotides were mixed in equimolar amounts directly before sample preparation for all experiments.

Capillary electrophoresis (CE) separations

Separations were carried out using a TSP-CE1000 capillary electrophoretic separation system (Thermo Separation Products, USA). Data were acquired and processed using OS/2 Warp v.3 software. Untreated fused silica capillaries (375 µm O.D. and 50 µm I.D.; Composite Metal Ltd, UK) were used with an effective length of 32 cm and a total length of 40 cm. The applied voltage was 25 kV and the capillary temperature was maintained at 20 ± 0.1°C. Oligonucleotides were detected and identified using high speed scanning in the UV region (200–350 nm) and the electropherograms were recorded at 260 nm. Buffer solutions were prepared by adjusting the pH of 0.02 M and 0.08 M sodium tetraborate solutions to 7.5 and 8.0 using 0.5 M boric acid, giving total borate concentrations (TBC) of 0.22 and 0.3 M. Sample and buffer solutions were prepared by milli-Q water purification (Millipore, Bedford, MA) and filtered through 0.2 µm pore size filters (Whatman International Ltd, UK). Free solution capillary electrophoresis (FSCE) showed our sample of AcD to be 87% pure, a figure which was used in subsequent calculations of binding ratios.

Competition experiments

Equimolar mixtures (10 pmol/µl) of each of the AT 12mer and GC 12mer single strands were incubated (1 h) with increasing concentrations of ligand, producing ligand to oligonucleotide (12mer duplex) ratios (*r*) in the ranges 1:0.0–1:7 for AcD, 1:1–1:15 for EtB and 1:1–1:5 for all minor groove binders (Dst, Nt and Ht). Electropherograms were obtained for all samples with a control sample (zero drug) injected before each run. All injections were made hydrodynamically for 5 s. For EtB, NaCl was added to give a final concentration of 0.02 M in the incubation mixture prior to electrophoresis.

Individual titration experiments

The individual oligonucleotide single strands were incubated for 1 h at room temperature with increasing concentrations of the drug and electropherograms obtained in the same way as for the competition experiments. All experiments were carried out in triplicate (at least) and average values were used to obtain the various plots. Peak height was used rather than peak area because there was not enough resolution to permit peak area quantification; calibration curves were constructed for samples containing increasing concentrations of equimolar mixtures of AT 12mer and GC 12mer to ensure the presence of a quantitative relationship between peak height and the amount of the oligonucleotide and were found to be linear in the range 2.5–20 pmol/µl. The relative standard deviations (RSD) for the peak height method ($n = 10$) of an equimolar mixture of AT 12mer and GC 12mer (at 2.5 pmol/µl) were found to be <5.2% for the AT 12mer and <6.6% for the GC 12mer.

RESULTS AND DISCUSSION

CE is a relatively new analytical technique which is based on the separation of analytes in microcapillaries (10–100 µm I.D.) under the influence of a high electric field. The advantages of CE include speed, quantification, use of an aqueous environment and low sample consumption. Several reports have described the use of CE as a method to study ligand–macromolecule interactions, including drug–DNA (14), drug–protein (15) and antigen–antibody interactions (16). There have been no published studies on the interaction of small molecules with DNA by FSCE.

In order to evaluate the suitability of the FSCE method, well-studied compounds representing the two main types of reversible interaction with DNA (intercalation and minor groove binding) were chosen. AcD, with definite specificity to sequences containing GC base pairs (17,18), and EtB were chosen as typical intercalators. Dst, Ht and Nt, which possess high specificity for AT-containing sequences (7,19), were chosen as minor groove binders.

Two non-self-complementary oligonucleotide duplexes were designed to examine the potential of the technique: d(AAATTA-TATTAT)·d(ATAATATAATTT) (AT 12mer) and d(GGGCCGC-GCCGC)·d(GCGGCGCGGCCC) (GC 12mer). The AT 12mer and GC 12mer were chosen so that: (i) one complete turn of DNA duplex was available for binding; (ii) the non-self-complementary nature of the oligonucleotide duplexes precluded the possibility of hairpin formation, which was found to complicate the analysis (unpublished data); and (iii) the oligonucleotides provided general GC and AT base pair combinations which served as templates for the sequence preference of DNA binding agents. A previously developed method (13) was found to be satisfactory for the separation of the AT 12mer and GC 12mer using borate buffer at pH 7.5 and 0.22 M TBC.

For four of the ligands studied (AcD, Nt, Dst and Ht) it was sufficient to prepare mixtures of the oligomer(s) and ligand in distilled water prior to electrophoresis: in general, peak shape is improved if the sample is of low ionic strength (20). Under these conditions the AT 12mer annealed only when bound to ligand (Nt, Dst or Ht). In the absence of a stabilizing ligand the single AT strands annealed on the column: variation in pH allowed the separation of excess single-strand from duplex, demonstrating duplex formation. UV spectra of eluted AT duplexes complexed with ligands (Fig. 5) were characteristic (21).

AcD did not complex with the AT 12mer, resulting in electropherograms in which the AT was unaffected by increasing concentrations of AcD. Addition of NaCl to the pre-column incubate to bring about AT annealing did not affect this result, the only difference being slight broadening of the oligomer peaks even in the absence of AcD. With EtB it was necessary to add NaCl to the pre-column incubate, otherwise there appeared to be no binding to the AT 12mer, which in distilled water would not anneal even in the presence of EtB.

Intercalators

Actinomycin D. Mixtures and individual samples of the AT 12mer and GC 12mer were titrated with AcD and electropherograms obtained at various drug to oligonucleotide ratios (*r*); selected electropherograms are shown in Figure 1. As expected (17,18), the electropherograms show selective binding to the GC 12mer with no sign of binding to the AT 12mer. This is inferred from the

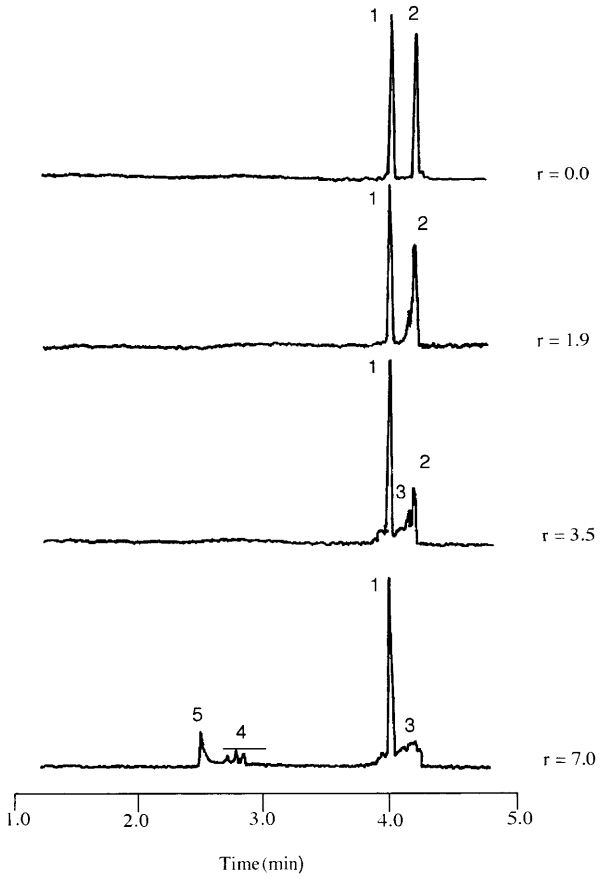

Figure 1. Electropherograms showing the competition experiment of AT 12mer and GC 12mer with actinomycin D. Ratios of AcD per 12mer duplex (*r*) are indicated. Peaks: 1, free AT 12mer; 2, free GC 12mer; 3, AcD–GC 12mer complex form(s); 4, AcD-related substances; 5, excess AcD. Buffer, 0.22 M TBC, pH 7.5.

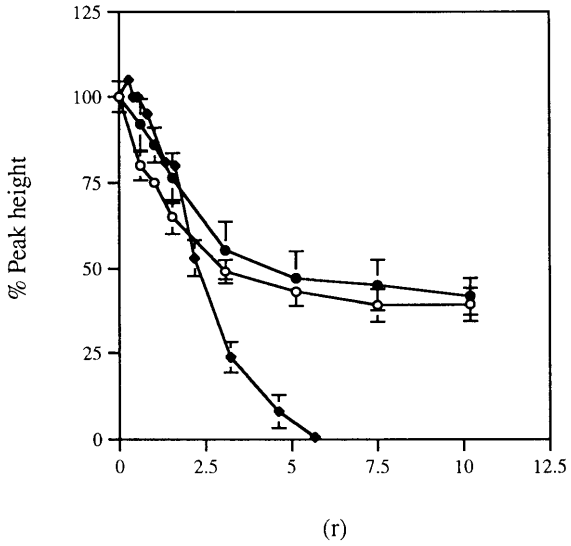

Figure 2. Binding isotherms of actinomycin D (◆) to GC 12mer and ethidium bromide to both AT 12mer (○) and GC 12mer (●) obtained by individual titration experiments. Peak height of the remaining GC 12mer as a percentage of that of a control sample is plotted against *r* (molar ratio of the drug per GC 12mer). The peak height of GC 12mer sample with no drug (injected immediately before the sample) was taken as 100%. Buffer, 0.22 M TBC, pH 7.5. Error bars show relative standard deviations. For EtB, NaCl (0.02 M) was added to all samples.

gradual disappearance of the GC peak and the emergence of a broad diffuse peak with a shorter migration time than that of the free GC peak. This broad peak was confirmed to be a complex of GC with AcD by UV spectral scanning. The broadness of the peak may be attributable to some dissociation of the complex during passage through the column and/or to different modes of binding.

In order to obtain quantitative binding data titration experiments were carried out for the GC 12mer with AcD. A plot of the percentage peak height of the GC 12mer compared with that of the GC 12mer in a control sample (no drug added) against *r* provides an estimate of the binding curve (Fig. 2), where *r* is the molar ratio of the added drug per GC 12mer duplex.

Given that the binding constant of AcD to a representative oligonucleotide was 1.5×10^7/M (22), it is reasonable to assume that all the added drug would be in the bound form up to the saturation point. The stoichiometry of binding can be directly determined from the binding isotherm at the complete disappearance of the free GC peak (Fig. 2), at ~*r* = 5. However, the broadness of the peak for the complex may indicate different affinities of binding for the five or more molecules of AcD which bind to each GC 12mer, with at least one AcD dissociating during electrophoresis. Possible sites are indicated by parentheses in the sequence : 5′-G()GG()CCG()C-G()CCG()C-3′. Of these, four are expected GC sites and one is an unusual GG site, for which there is a precedent (23). Assuming five binding sites, probability theory predicts that at 40% occupancy there should be 8% free oligomer. Experiment (Fig. 1) shows 57% free oligomer at 38% occupancy (*r* = 1.9), showing a high degree of cooperativity in the binding of AcD to this base pair sequence. The GC 12 mer used in the present study was designed to test the FSCE method: more detailed information about AcD binding could be gained from oligomers with fewer binding sites.

Ethidium bromide. The data for EtB were treated in the same way as for AcD. Earlier studies on EtB reported the compound to have no definite sequence preference (24). However footprinting at 4°C induced marked changes in the pattern of cleavage (10) and the weakest binding was observed for poly(dA) sequences. In the present study, at 20°C, there was no clear preference (Fig. 2).

The binding curve for EtB plateaus above five molecules of EtB to both GC and AT 12mers (Fig. 2). At $r > 5$ no further changes were observed for the oligomer peaks and excess free EtB started to appear in the electropherogram (Fig. 3). There are two notable differences between the electropherograms for AcD and EtB. With AcD, complexes were formed which were sufficiently stable to reach the detector, with different migration times from the free oligomer, so that the height of the free oligomer peak reached zero (Fig. 2) in the presence of sufficient ligand. With EtB, the complex did not survive to reach the detector; dissociation occurred on-column, resulting in peak broadening for both oligomer and ligand. Thus, a clear peak for free EtB only appears above the concentration required to saturate the oligomer (Fig. 3). Beyond this point, there is no further peak broadening effect on the oligomer and the oligomer peak height plateaus at a non-zero value (Fig. 2).

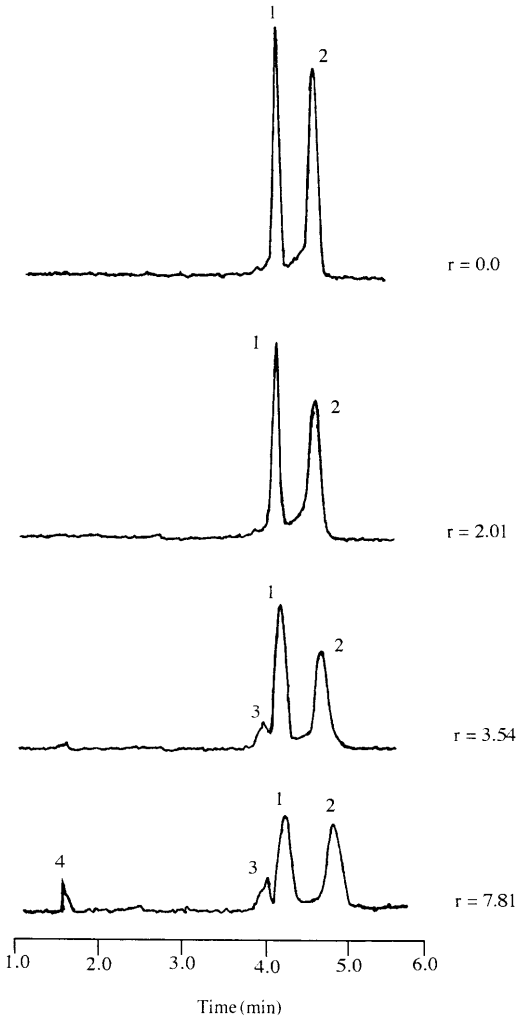

Figure 3. Typical electropherograms for equimolar mixtures of AT 12mer and GC 12mer incubated with increasing concentrations of ethidium bromide. Ratios of EtB per oligonucleotide 12mer duplex (*r*) are indicated. Peaks: 1, AT 12mer; 2, GC 12mer; 3, unknown; 4, excess EtB. Buffer, 0.22 M TBC, pH 7.5.

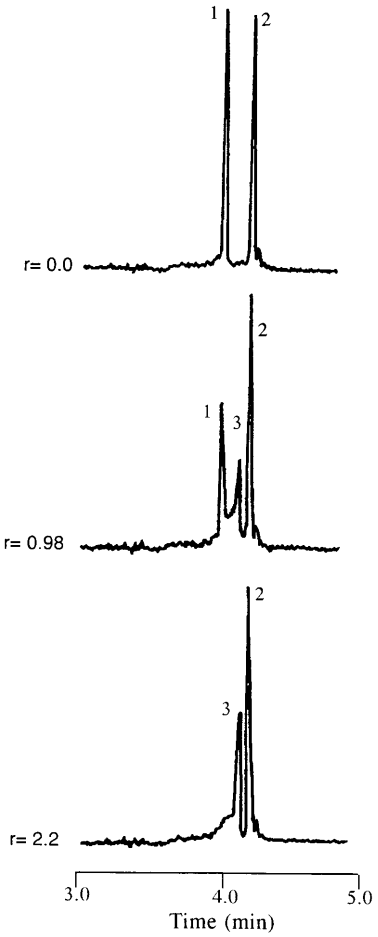

Figure 4. Typical electropherograms for equimolar mixtures of AT 12mer and GC 12mer incubated with increasing concentrations of netropsin. Ratios of netropsin per oligonucleotide 12mer duplex (*r*) are indicated. Peaks: 1, free AT 12mer; 2, free GC 12mer; 3, netropsin–AT 12mer complex. Buffer, 0.22 M TBC, pH 7.5.

Minor groove binders

The competition experiment for the oligonucleotide mixture with the minor groove binder Nt shows a clear preference for the AT 12mer over the GC 12mer (Fig. 4), as seen from the gradual decrease in the peak area (and height) of the AT 12mer and the appearance of an AT 12mer–drug complex peak until no further AT 12mer remains, while the GC 12mer is not altered. In order to make sure that the GC 12mer did not form complexes which were not separated from the free GC 12mer, UV spectra were obtained at different points on the GC 12mer peak (i.e. peak slicing). These spectra were identical and characteristic of the GC 12mer, indicating the presence of only one species. Since Nt and Dst complexed to DNA are known to exhibit UV maxima at ~320 nm (21), the ability to obtain UV spectra of the eluting peaks is a powerful tool for examining complex formation.

The AT 12mer–Nt complex appears in Figure 4 as a well-defined peak, with a longer migration time than the free AT 12mer, although not separated to baseline. Attempts to achieve complete separation of the complex by changing the concentration of the buffer and/or the pH (7.5–9.0) enhanced the resolution between the free AT 12mer and GC 12mer, but only brought about a slight improvement in the separation of the free and complexed forms of the AT 12mer.

Similar electropherograms to that shown in Figure 4 were obtained for titration of the oligonucleotide mixture with Ht and Dst, showing the clear preference of the drugs for the AT 12mer. In each case the new peak, which had a migration time longer than the free AT 12mer, was confirmed to be the complexed form of the AT 12mer (with either Nt, Dst or Ht) by obtaining the UV spectra (Fig. 5), which showed the characteristics of minor groove binder–DNA complexes (21).

The experiment was repeated with the AT 12mer and GC 12mer separately. In order to obtain quantitative binding data the peak height of the remaining (free) AT 12mer as a percentage of the control AT 12mer (zero drug) was used as a measure of the

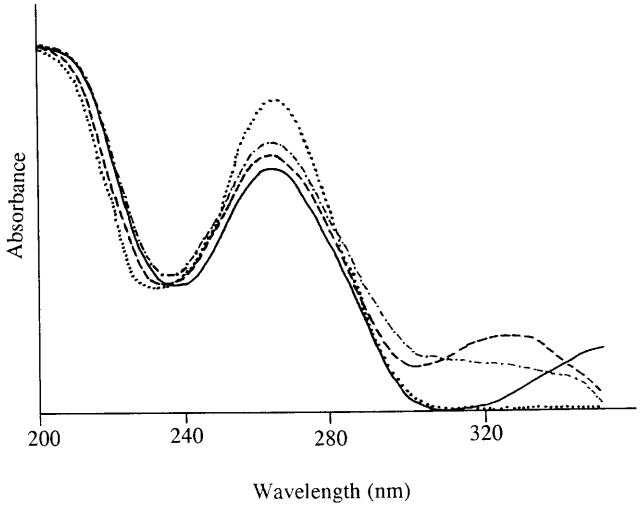

Figure 5. Normalized UV spectra of free AT 12mer (...) and AT 12mer complexed to netropsin (-·-·-), distamycin (- - -) and Hoechst 33258 (—). Buffer, 0.22 M TBC, pH 7.5.

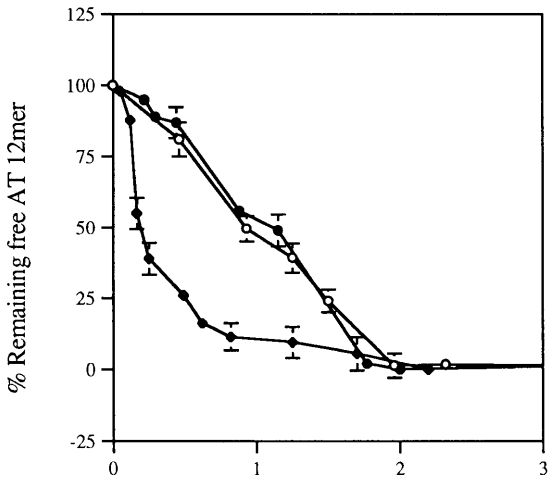

Figure 6. Binding curves of netropsin (○), distamycin (◆) and Hoechst 33258 (●) obtained by titrating AT 12mer with each of the drugs separately. Peak height of a control sample (zero drug) was taken as 100%. Buffer, 0.22 M TBC, pH 7.5. Error bars show relative standard deviations.

remaining free oligonucleotide. A calibration curve was constructed using solutions of equimolar mixtures of AT 12mer and GC 12mer in a similar way to that for EtB and AcD. Binding curves were obtained by plotting the peak height of the AT 12mer in the sample (at each r value) divided by the peak height of the AT 12mer control ($r = 0$), which was injected immediately before the sample, against the molar ratio (r) of the added drug per 12mer (Fig. 6). Since all three minor groove binders (Ht, Nt and Dst) bind strongly to AT-containing oligonucleotides ($K_a = 1 \times 10^{-6}$ to 10^{-9}/M) (25,26), the unbound drug at each of the titration points is negligible, so that the stoichiometry of binding can be obtained directly from the binding curves. The binding curves in Figure 6 show a binding stoichiometry of two drug molecules per AT 12mer for each of the three compounds at saturation, which is in very good agreement with the literature data (27,28). However, the plot in Figure 6 shows different binding isotherms for Nt, Ht and Dst.

Netropsin (Nt) and Hoechst 33258 (Ht). For Nt and Ht the binding isotherms were essentially linear (Fig. 6), which suggests one mode of binding in a cooperative manner. The cooperativity of binding is evident from the binding curve, since at $r = 1$ there was only 50% of the AT 12mer in the bound form. The binding of Nt and Ht to the AT 12mer occurs only in the 2:1 mode, i.e. binding of the first drug molecule facilitates binding of the second. Using NMR techniques it has been demonstrated that Ht binds cooperatively to a 12mer oligonucleotide with two separate binding sites (28). The literature evidence for cooperative binding of Nt is indirect, since there have been no binding studies with similar oligonucleotides containing only two separate binding sites (29).

Distamycin (Dst). For Dst the binding curve was non-linear (Fig. 6), with a saturation binding ratio of 2:1. Unlike Nt and Ht, at $r = 1$ there was >90% of the AT 12mer in the bound form. In this case it is evident that 1:1 binding occurs at lower r values (<1) and 2:1 binding occurs at $r > 1$.

Since Dst requires a binding site of 4–6 bp (27,30,31), the AT 12mer can accommodate two Dst molecules (possibly separated by 1–3 bp). All of the reported studies on Dst–oligonucleotide complexes were performed with oligonucleotides containing single binding sites or with polynucleotides that contain multiple binding sites (29,32).

It is well established that Dst can bind to single binding sites of at least five AT bp in 2:1 (Dst:oligonucleotide) mode (29). In these 2:1 complexes the two Dst molecules bind side-by-side (head-to-tail) in the minor groove of the AT sequence. Molecular mechanics calculations indicate that the minor groove must expand significantly to accommodate two drug molecules side-by-side (33). Since there have been no studies on the binding of Nt and Dst to 12mer oligonucleotides with AT combinations having two potential binding sites, it would be interesting to observe the structural detail of the complex at the molecular level using X-ray or NMR techniques. The present study does not permit a distinction to be made between linear and side-by-side binding.

CONCLUSIONS

CE has been successfully used to probe the sequence preference of DNA binding agents, including minor groove binders and intercalators. In agreement with previous studies, the CE method showed the preference of AcD for a GC-containing oligonucleotide and the preference of the minor groove binders Nt, Dst and Ht for AT-containing sequences, while EtB showed no significant preference.

Since FSCE can separate the free and bound ligands and/or the free and bound oligonucleotides, it is possible to obtain estimates of binding curves and binding ratios. Cooperativity of binding can be directly probed by observing the change in peak height of the free oligonucleotide as a function of drug concentration, and the method showed such binding of Ht to an AT 12mer in agreement with a previous NMR study (28). CE showed cooperative binding of Nt to an AT 12mer and non-cooperative binding of Dst to the same AT 12mer.

An important feature of FSCE is the dependence of the migration time on the conformation of the oligonucleotide or oligonucleotide–drug complex (13). Since intercalating drugs produce changes in DNA secondary structure which are different from those induced by minor groove binders, a systematic difference in the migration time might be expected for intercalators and minor groove binders, provided that the complex survives as a single species during electrophoresis. Such differences in electrophoretic behaviour between minor groove binders and intercalators could provide empirical evidence of the mode of binding of newly designed DNA binding molecules.

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