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A surface plasmon resonance method for detecting multiple modes of DNA–ligand interactions

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Abstract

A simple and general surface plasmon resonance (SPR) based method has been developed to detect and quantitate binding of low molecular weight compounds (200–1200 Da) to double stranded DNA. Several compounds were chosen to probe three different modes of binding interactions, intercalation, minor groove binding and electrostatic interactions. Ethidium bromide (MW 390 Da), a probe of intercalative binding, was tested by plotting the steady state SPR responses measured on a DNA modified surface versus ethidium bromide concentration. The best fit of the binding isotherm gave a K_{eq} of 1.8×10^5 M^{-1}. Co-solvents such as DMSO are often used in activity assays to increase the solubility of poorly water-soluble drugs. The effect of DMSO on the ethidium bromide/DNA interaction was also tested by measuring binding in the presence of 0, 1 and 5% DMSO. No effect on the measured K_{eq} was observed at these DMSO concentrations. The binding of actinomycin (MW 1255 Da), an antibiotic known to bind DNA through intercalation and minor groove binding, was also tested. The K_{eq} estimated from the steady state responses on a DNA surface was 1.9×10^6 M^{-1}. DAPI (MW 350 Da) (4′,6-diamidino-2-phenylindole) a fluorescent probe which binds the minor groove of DNA was also tested and gave a K_{eq} of 1.8×10^6 M^{-1} measured by SPR. Finally, spermine (MW 202) a compound known to bind DNA through ionic interactions gave the weakest K_{eq} of 1.7×10^4 M^{-1}. All the K_{eq} values measured by SPR and reported for these compounds were in good agreement with literature values measured by other techniques.

Keywords: DNA–ligand interactions; Surface plasmon resonance; Resonance units

1. Introduction

The identification of compounds which can interfere with gene expression is important in the discovery of novel anti-viral and anti-tumoral agents. Analytical techniques, which can rapidly and easily identify potential therapeutics are important to the drug discovery and development

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processes. DNA–drug interactions can be studied by a wide range of established techniques. These include spectrophotometry [1], equilibrium dialysis, thermal denaturation, DNA footprinting and ultracentrifugation [2]. These techniques generally involve the use of labeled reagents or are limited to molecules with specific spectrophotometric characteristics or require a binding induced change in the physical properties of the DNA. For example, spectrophotometric methods require a perturbation in the UV–vis absorption spectrum of the drug molecule. In situations where a spectrophotometric change is not measurable, equilibrium dialysis can be used; however, a radiolabeled compound is needed. DNA footprinting assays can be disadvantageous when large numbers of compounds need to be tested since electrophoretic mobilities are determined on polyacrylamide gels which have limited sample number capacity. Quantitation of binding constants is difficult by this method since it requires optimization of DNA digestion conditions and data interpretation can be complicated by uneven loading of labeled probes on the gel [3]. A general method capable of label free detection of different types of DNA–ligand interactions would be useful, especially when screening large numbers of compounds with a high degree of chemical diversity.

One approach to understanding the nucleic acid binding properties of low molecular weight (LMW) compounds is to use surface plasmon resonance to detect binding of compounds to an immobilized double stranded DNA (dsDNA) target. Surface plasmon resonance techniques measure small local changes in refractive index near a surface [4]. The SPR signal (expressed as resonance units, RU) correlates to the surface concentration of a ligand bound to the sensor chip surface. The technique has been used to measure binding affinities between DNA immobilized on a surface and proteins in solution [5], binding of the drug chromomycin to target DNA sequences [6] and to identify small molecules from a aminoglucopyranoside based library that recognize RNA [7]. This report describes an SPR based method for measuring small molecule/DNA interactions by utilizing a 5′-biotinylated 47 base pair DNA molecule immobilized on the surface of a streptavidin coated sensorchip. The advantage of an SPR based method is that it provides a direct, one step, label free assay for measuring drug/DNA interactions.

As part of developing a reliable and robust screening assay for detecting a broad range of small molecule interactions with DNA, some well-known and well-characterized DNA binding molecules were tested. Four compounds were chosen because they encompass most of the common ligand/DNA interaction modes (e.g. intercalation, minor groove binding and ionic interactions). These compounds included actinomycin D, ethidium bromide, DAPI (4′,6-diamidino-2-phenylindole) and spermine. Actinomycin D, an anti-tumor antibiotic, contains a 2-aminophenoxazin-3-one chromophore and two cyclic pentapeptide lactones. The biological activity of actinomycin D is believed to be due to its ability to bind to duplex DNA and thereby inhibit DNA-dependent RNA polymerase. Previous work suggests that actinomycin interacts with duplex DNA both through intercalative binding and minor groove binding [8].

Ethidium bromide, a well studied DNA intercalator, was also tested in these experiments, as was DAPI (4′,6-diamidino-2-phenylindole dichloride) an apparent minor groove binder to A–T rich sequences of DNA [9]. Finally, the polyamine spermine was studied to characterize charge-related interactions with DNA. Structures of the compounds are shown in Fig. 1.

2. Materials and methods

2.1. DNA binding compounds

All compounds were purchased from commercial sources and used without further purification. Ethidium bromide, spermine tetrachloride, and DAPI (4′,6-diamidino-2-phenylindole) were purchased from Sigma Aldrich (St. Louis, MO). Actinomycin was purchased from Molecular Probes (Eugene, OR). Stock solutions of ethidium bromide (0.2 M in HBS buffer which consists of 10 mM HEPES, 150 mM NaCl, 3 mM EDTA,

0.005% v/v polysorbate 20, pH 7.4), actinomycin (12 μM in water), spermine (0.2 M in HBS), and DAPI (1 mM in DMSO) were frozen at −20°C until needed for use. Concentrations for ethidium bromide, actinomycin, and DAPI were verified spectrophotometrically by UV–vis absorption.

2.2. DNA substrate

The oligonucleotide with sequence 5′-biotin-TAGTACCGCCACCCTCAGAACCGTTATTGCATGAAAGCCCGGCTGCC and its complement were synthesized and HPLC purified by Genosys Biotechnologies Inc. (The Woodlands, TX). The oligonucleotides were supplied as a dry powder and were reconstituted to ~20 μM with 10 mM Tris, 1 mM EDTA, pH 7.4 and stored at −20°C until needed for use. The single-stranded oligonucleotide shown above was biotinylated at the 5′ end to facilitate immobilization to the biosensor chip surface.

The oligonucleotides were annealed by heating 100 μl of a 2 μM solution of each of the oligonucleotides to 80°C for 10 min. Oligonucleotides were diluted using 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20, pH 7.4. The two solutions were then combined and allowed to cool at room temperature. DNA annealing was confirmed by gel electrophoresis (data not shown).

2.3. Experimental assay procedure using surface plasmon resonance

Surface plasmon resonance measurements were made using a BIAcore™ 2000 instrument (Biacore

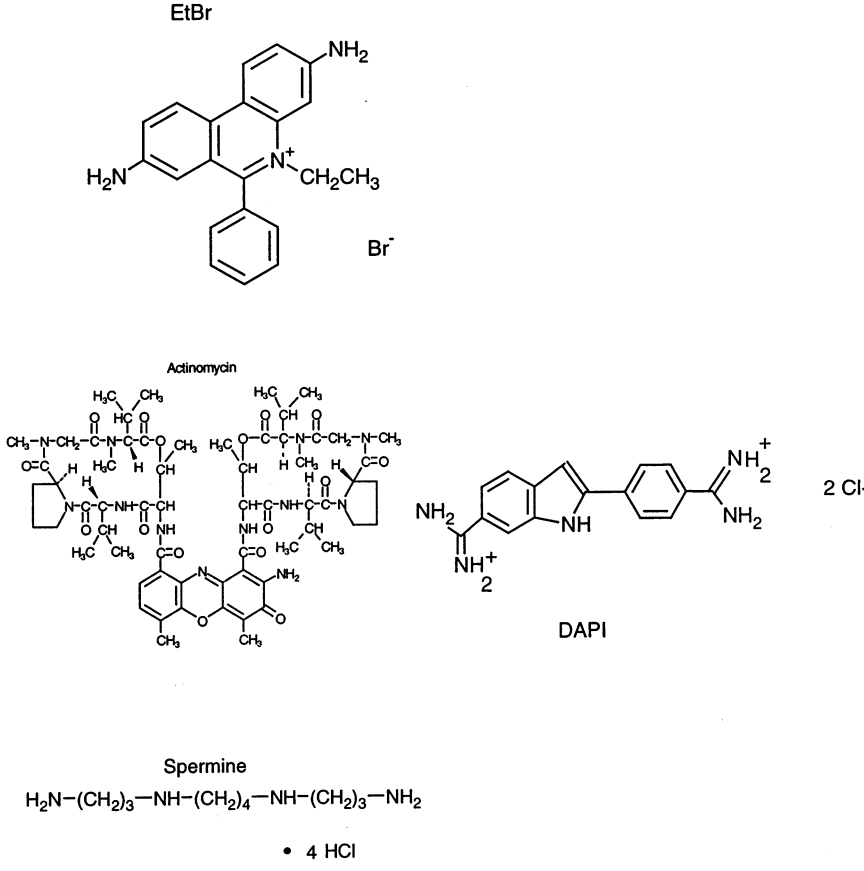

Fig. 1. Chemical structures of LMW DNA binding molecules.

Inc., Upsaala, Sweden). The dsDNA probe was captured onto a streptavidin biosensor chip surface (SA5 chip, Biacore Inc., Upsaala, Sweden) by first preconditioning all four flowcells on the chip surface with three consecutive 1-min pulses of 1 M NaCl in 50 mM NaOH. Immobilization of dsDNA was achieved by injecting a 2-μM solution of dsDNA for 6 min at a flowrate of 5 μl min^{-1} over one streptavidin flowcell. All binding measurements with small molecules, unless otherwise noted, were performed in a buffer composed of 10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20, pH 7.4. Binding interactions were measured at a flowrate of 5 μl min^{-1} and at a constant temperature of 25°C. Injections were made simultaneously over all four flowcells. In a typical experiment specific interactions with dsDNA were detected on flowcell #2 and nonspecific interactions with streptavidin only were detected on flowcell #1. In some experiments, streptavidin was chemically linked to a conventional CM5 sensorchip using *N*-hydroxysuccinimide/*N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide coupling chemistry as described by Biacore Inc. [4]. In those experiments binding of LMW compounds to DNA was background subtracted using a blank (EDC/NHS activated/ethanolamine deactivated) flowcell because it represented a non-protein modified surface. In most experiments, association of LMW compounds was measured for 6 min followed by 5 min of dissociation. The surface was regenerated with a 5 μl pulse of 0.1% SDS at 100 μl min^{-1}.

2.4. Data analysis

Experimental data were quantitated by measuring the signal response, after background subtraction at the signal plateau or steady state level, typically very near the end of the analyte injection. The steady state response was then plotted versus the analyte concentration and the data were fit to a simple Langmuir isotherm for bimolecular interactions [10]:

$$R_{eq} = R^{*}_{max}\left\{\frac{K_{eq}[A]}{1 + K_{eq}[A]}\right\} \quad (1)$$

where R_{eq} is the measured response, [A] is the concentration of solution ligand, K_{eq} is the apparent equilibrium constant, and R_{max} is the maximum response at saturation of surface binding sites. The free parameters are R_{max} and K_{eq}. Values of R[A] are obtained at a series of injected analyte [A] solution concentrations. The data were curve fit using Sigma Plot™ (Jandel Scientific, Carle Mader, CA).

The measured SPR response in RUs was assumed to correlate to the surface concentration of immobilized DNA and surface bound small molecule using the estimate 1 RU ≅ 1 pg mm^{-2} as has been done in the past for proteins and oligonucleotides [11]. Because of this, the R_{max} value can be used to calculate a maximum binding ratio (mbr), assuming one set of independent noninteracting binding sites, using the following equation:

$$\text{mbr} = \frac{R_{max}}{\text{MW ligand}} \frac{\text{MW DNA substrate}}{\text{DNA}_{surf}} \frac{1}{47} \quad (2)$$

where DNA_{surf} is the amount of surface immobilized DNA in RUs and 47 is the number of base pairs.

3. Results and discussion

3.1. Immobilization of 47 base pair DNA probe

Streptavidin coated and blank flowcells were exposed to a 2 μM solution of HPLC purified biotinylated 47 bp DNA. A time-dependent, stable immobilization of DNA was observed (Fig. 2) on the streptavidin surface. The large negative change in refractive index during immobilization is due to differences in buffer compositions in the DNA sample and the assay running buffer. Immobilization was not observed on the blank flowcell. Typical immobilization levels for the 47 bp DNA probe were between 500 and 3000 RUs which corresponds to 0.02–0.1 pmole DNA per mm^2.

3.2. Interactions of ethidium bromide with dsDNA probe

Ethidium bromide (EtBr MW 390 Da) interca-

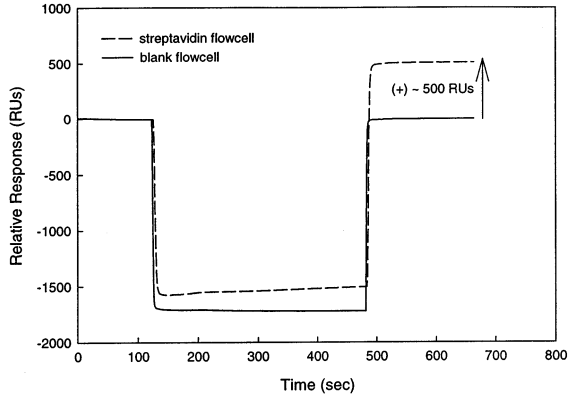

Fig. 2. A simultaneous injection of (2 μM) dsDNA over blank (—) and streptavidin flowcells (– –) illustrates capture of biotinylated 47 bp dsDNA on a streptavidin flowcell.

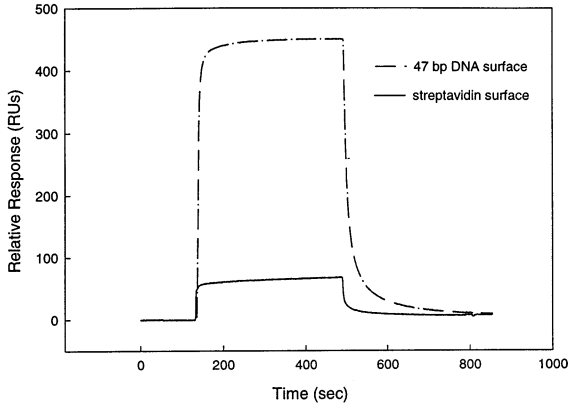

Fig. 3. A 15 μM solution of EtBr in HBS buffer is injected over a DNA (—·—) and streptavidin (—) surface simultaneously.

lates both double stranded and single stranded DNA at micromolar concentrations [12]. In this work, ethidium bromide was found to preferentially bind to a 47 bp DNA surface when a 15 μM solution of EtBr was injected over DNA (~ 3000 RUs) and streptavidin surfaces simultaneously (Fig. 3).

The binding of EtBr to the 47 bp DNA probe was measured at concentrations ranging from 0.12–15 μM. Bromphenol blue (MW 692 Da) was used as a negative control for EtBr because of its similar molecular weight. No binding of bromphenol blue to DNA was observed at similar concentrations (data not shown). The steady state binding response measured at 342 s after the injection was plotted versus EtBr concentration (Fig. 4). The binding isotherm was fit to a simple 1:1 binding model Eq. (1) yielding a K_{eq} of 1.8×10^5 M^{-1}. This value is in good agreement with K_{eq} values published for EtBr binding to other DNAs (2.6×10^5 M^{-1} [13]; 1.5×10^5 M^{-1} [12]).

The binding ratio estimated at maximum saturation for EtBr binding to immobilized DNA was 0.26 or ≈1 drug/4 bp. It is known in the literature that ethidium bromide binds to DNA at a ratio of 1 drug/2 bp or 0.50, when fully saturated following an 'excluded site' binding model [13]. Possible explanations for the lower binding ratio measured by SPR are that the binding of EtBr to immobilized DNA in this experiment is more restricted, possibly due to the surface immobilized presentation of DNA. Also it is known that EtBr binding induces DNA to increase in length by slight unwinding of the double helix when fully saturated, perhaps the orientation of the DNA on the sensorchip surface hinders this physical change in the DNA structure. These data suggest that the SPR method may underestimate the bind-

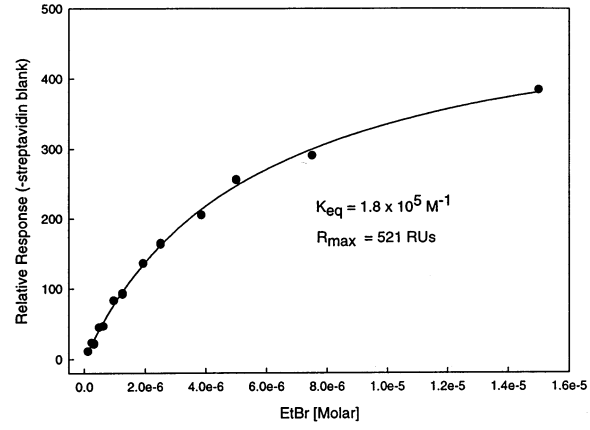

Fig. 4. Steady state binding response versus concentration for EtBr binding to the 47 bp DNA surface. EtBr concentrations ranged from 0.12 to 15 μM. The DNA surface immobilization level was ≈3300 RUs.

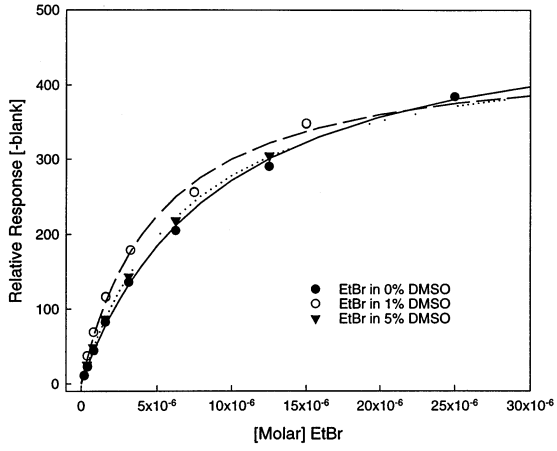

Fig. 5. Binding of EtBr to DNA was tested in the presence of 0% (●), 1% (○) and 5% (▼) DMSO to assess the effect of the solvent on binding interactions.

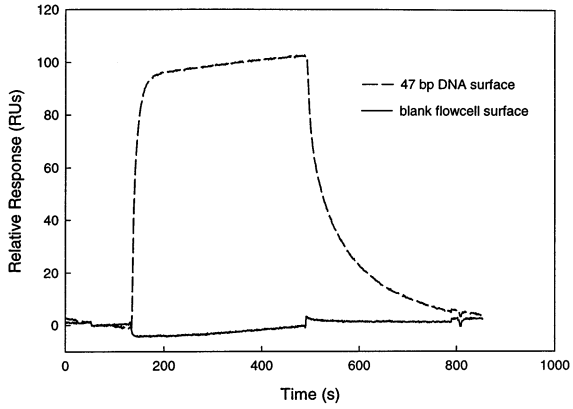

Fig. 6. A 3.3 μM solution of actinomycin in HBS buffer is injected over a DNA (– – –) and blank (—) surface simultaneously.

ing ratio for compounds that bind DNA through intercalation, however the nature of this effect was not probed further.

3.3. Testing the effects of DMSO on EtBr/DNA binding

Very often dimethyl sulfoxide is used to solubilize compounds for assessment of activity in drug discovery screening assays. Certain co-solvents such as DMSO can cause large bulk refractive index changes. As a test of assay ruggedness, it was desirable to determine if DMSO had a negative impact on EtBr/DNA binding. This was tested by adding 0, 1, and 5% DMSO into the running buffer and dilution buffers (Fig. 5). The following parameters were obtained from fitting the binding curves shown in Fig. 5, 0% DMSO ($K_{eq} = 1.1 \times 10^5$ M^{-1}, $R_{max} = 519$ RUs); 1% DMSO ($K_{eq} = 2.0 \times 10^5$ M^{-1}, $R_{max} = 450$ RUs); 5% DMSO ($K_{eq} = 1.4 \times 10^5$ M^{-1}, $R_{max} = 477$ RUs). The K_{eq} and R_{max} values suggest that DMSO concentrations up to 5% have little effect on the affinity or maximum saturation levels of EtBr binding to the 47 bp DNA. A significant increase in the bulk refractive index effect with increasing DMSO concentration was observed which made background subtraction of binding data more difficult. This factor needs to be considered when compounds containing DMSO are analyzed.

3.4. Interactions of actinomycin with dsDNA probe

Actinomycin (MW 1255) was found to preferentially bind a DNA surface when a 3.3 μM solution was injected simultaneously over DNA modified and blank flowcells (Fig. 6). The binding of actinomycin to the 47 bp DNA probe was measured at concentrations ranging from 0.82 to 6.6 μM (Fig. 7). Actinomycin is not significantly

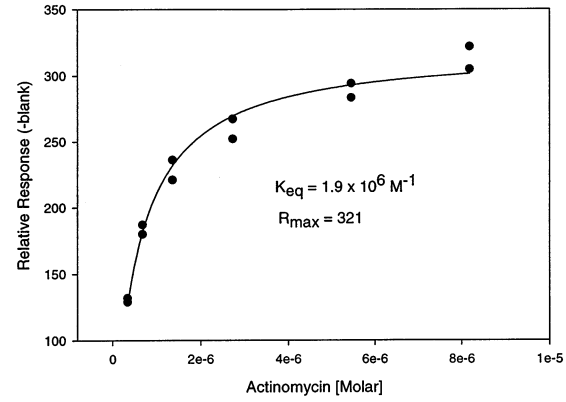

Fig. 7. Steady state binding response versus concentration for actinomycin binding to the 47 bp DNA surface. Actinomycin concentrations ranged from 0.82 to 6.6 μM. The DNA surface immobilization level was ≈1126 RUs.

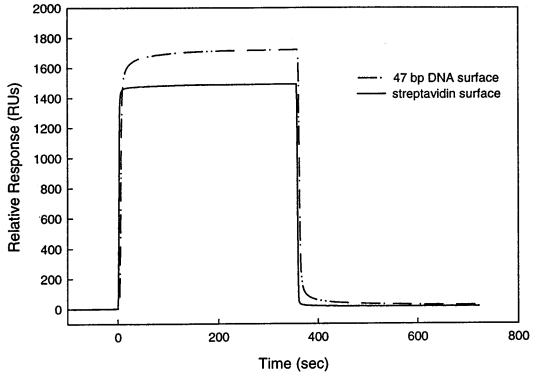

Fig. 8. A 1 μM solution of DAPI in HBS buffer + 1% DMSO is injected over a DNA (—· ·—) and streptavidin (—) surface simultaneously.

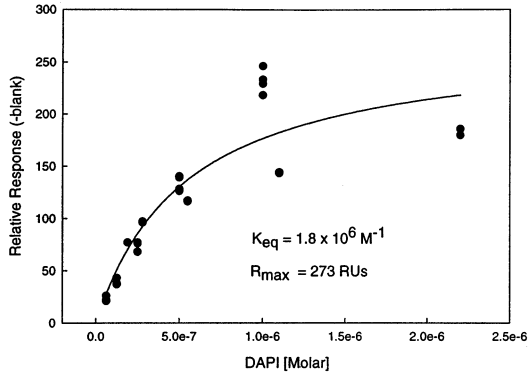

Fig. 9. Steady state binding response versus concentration for DAPI binding to the 47 bp DNA surface. DAPI concentrations ranged from 0.06 to 2 μM. The DNA surface immobilization level was ≈3000 RUs.

soluble in water, as a result concentrations greater than 12 μM could not be attained. Analysis of the binding curve yielded $K_{eq} = 1.9 \times 10^6$ M^{-1} and $R_{max} = 321$ RUs. The binding constant is in reasonable agreement with the range of values found in the literature ($5–12 \times 10^6$ M^{-1} [8]).

Actinomycin is known to intercalate and bind strongly to G–C containing sites on DNA [8,14]. The 47 bp DNA probe used in these experiments contains five G–C sites. The maximum binding ratio (actinomycin molecules per bp) was calculated using the R_{max} from the best fit of the actinomycin binding curve (Fig. 7) and the known amount of DNA immobilized. The binding ratio was 0.14 or ≈1 drug/4 bp. If actinomycin bound to all G–C sites present in the DNA probe then the binding ratio would be expected to be ∼0.1. Since the estimated binding ratio is close to 0.1 it may suggest that binding is occurring at G–C sites.

3.5. Interactions of DAPI with dsDNA probe

A 1 μM injection of DAPI (MW 350.2) was found to preferentially bind a DNA coupled flowcell versus a blank flowcell as shown in Fig. 8. A steady state plateau was reached after ∼5 min into the injection. There was a significant bulk refractive index effect due to the presence of 1% DMSO in the diluent and running buffer. The DMSO was included to increase the solubility of DAPI, which is not very water soluble. The steady state binding responses were plotted versus concentration for DAPI concentrations between 0.06 and 2 μM (Fig. 9). Analysis of the binding curve yielded a $K_{eq} = 1.8 \times 10^6$ M^{-1} and $R_{max} = 273$ RUs. This value is in good agreement with other reported binding constants for DAPI and nucleic acids ($10^5 – 10^7$ M^{-1} [9]). The maximum binding ratio (DAPI molecules per bp) was calculated using the R_{max} from the best fit of the DAPI binding curve (Fig. 9) and the known amount of DNA immobilized. The binding ratio was calculated to be 0.16. This value is in good agreement with binding ratios measured previously [15].

3.6. Interactions of spermine with dsDNA probe

Spermine (MW 202.34) was found to interact weakly with DNA (Fig. 10). Shown in Fig. 10 is an injection of 100 μM spermine. A large bulk refractive index effect was observed at this concentration which looked similar in shape to injections of compounds that do not bind DNA. To verify that a specific interaction with DNA was occurring, spermine was injected over a blank, 200 RU DNA surface, and a 3000 RU DNA surface. The steady state signal amplitude increased with the amount of DNA on the surface indicating that specific binding was occurring. In general, responses were seen only at spermine concentrations greater than 6.3 μM. The steady

state responses versus spermine concentration are shown in Fig. 11. Fitting of the binding isotherm yielded the following results, $K_{eq} = 1.7 \times 10^4\ M^{-1}$ and $R_{max} = 101$ RUs. The K_{eq} for spermine is much weaker than both the intercalating dyes and minor groove binders ($11–0.8 \times 10^3\ M^{-1}$ [16]). The K_{eq} value obtained by SPR is in good agreement with other K_{eq} values determined for spermine/DNA binding by equilibrium dialysis studies.

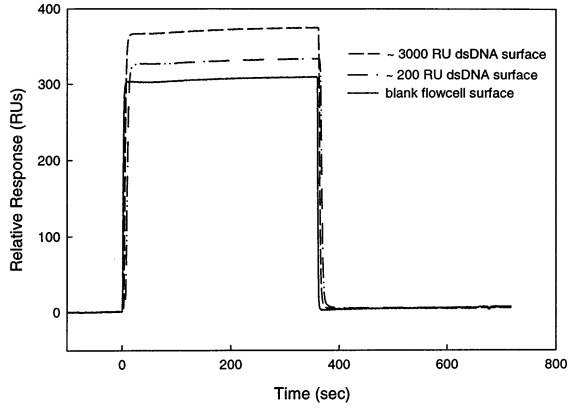

Fig. 10. Simultaneous injection of 100 μM spermine in HBS buffer over a blank (—), 200 RU (—· ·—) and 3000 RU (– – –) DNA surface.

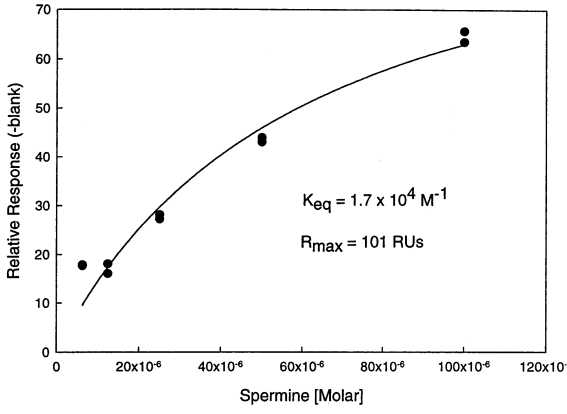

Fig. 11. Steady state binding response versus concentration for spermine binding to the 47 bp DNA surface. Spermine concentrations ranged from 6.3 to 100 μM. The DNA surface immobilization level was ≈3000 RUs.

4. Conclusions

The use of SPR for detection of small molecule binding is technically challenging because refractive index changes caused by LMW compounds are normally low, in the range of 10–100 RUs. Additionally the small molecule is often present in 1–10% DMSO to increase its solubility, however, even low amounts (1% or less) of DMSO cause very large bulk refractive index effects which are detectable by SPR. This work demonstrates that SPR can successfully detect and quantitate binding of small molecules to DNA by three different mechanisms, intercalation, minor groove binding, and electrostatics. The binding constants measured ranged from 10^4 to $10^6\ M^{-1}$. This methodology may prove to be useful in drug development and serve as an assay for DNA binding ability. Novel quantitative kinetic data and equilibrium constants can be determined using SPR and should be exploited in the future.

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