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Fluorometric Broad-Range Screening of Compounds with Affinity for Nucleic Acids

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The potential of a nucleic acid-based optical bioprobe for environmental measurements and drug monitoring is described. The sensor employs the long-wavelength intercalating fluorophore TO-PRO-3 (TP3). Compounds that interact with the TP3−DNA complex are indirectly detected by a decrease in the fluorescence intensity. We found that the configuration and length of the DNA dramatically affected the intensity of the fluorescence emitted from the TP3−DNA complex. We compared nucleic acids from different sources and optimized the system for pBR322 plasmid DNA (4363 bp) digested by *Hin*dIII restriction endonuclease. This endonuclease has a single recognition site in plasmid pBR322. In the proposed method, we attempt to combine broad-range detection with rapid and simple operation. A fiber-optic capillary fluorescence system was used to analyze toxic aromatic amines, antibiotics, and several kinds of antitumor drugs, using small amounts of sample, down to 10 μL, with a sensitivity comparable to that of current electrochemical methods. The detection limit can be as low as a few ppb or submicromolar. This approach is useful for routine screening in environmental monitoring or for controlling cytotoxic drug administration. The ease of operation and the rapid response allow high-throughput screening.

In recent years, there has been an enormous increase in the use of nucleic acids as a tool in the recognition or monitoring of chemical compounds of environmental interest that may affect the genome. Nucleic acids have many unique structural features that offer reliable means of recognizing and monitoring many compounds of medical or environmental importance, and major research activities in these areas have been directed toward the design of sequence-selective biosensors based on hybridization.[1] Commonly used methods for identifying carcinogens include epidemiology, mammalian bioassays, and rapid assays, such as low-temperature laser-induced fluorescence detection, which was recently suggested for the analysis of DNA−carcinogen interactions.[2,3] A simple method for the detection of mutations in DNA oligonucleotides using double-stranded DNA (dsDNA) and a specific nucleic acid intercalator dye has already been described,[4] and a strategy was proposed for the detection of compounds with affinity for nucleic acids. At the same time, an electrochemical method for the same purpose was introduced.[5−7]

Here, we report on the development of a method to detect the interaction of compounds with nucleic acids through changes in the fluorescence intensity of the fluorescent intercalator TO-PRO-3 (TP3), a monomeric thiazole orange derivative.[8] The intercalation process reflects the ability of a planar aromatic or heterocyclic aromatic system to become inserted between contiguous base pairs of a nucleic acid molecule without disturbing the overall stacking pattern[9] or requiring covalent modification. Our development considerably decreases the volume of sample/reagent required (total volume 10 μL) compared to previous procedures, increases the sensitivity, and facilitates high-throughput microchip screening applications. TP3 is a cyanine monomer dye that specifically binds with dsDNA and is frequently used in flow cytometric analysis. TP3 has a very high binding affinity to dsDNA, with dissociation constants in the micromolar range, but without sequence selectivity.[10] When TP3 binds to a dsDNA, it exhibits increased fluorescence. Binding of certain compounds to the nucleotide induces a partial block of the duplex in the DNA sequence, which leads to a decrease in the degree of intercalation of TP3. The displacement of the dye by another compound with affinity for the nucleic acid can thus be detected as a decrease in the fluorescence intensity of TP3. The TP3−DNA complex emits long-wavelength emission after excitation (642/661 nm), in a wavelength range with low autofluorescence from natural compounds, which enhances the signal/noise ratio.

The aim of this study was to further explore the possibility of using this approach for broad-range screening of compounds with

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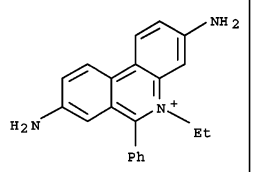
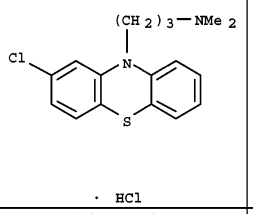
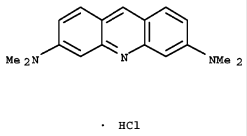
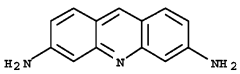
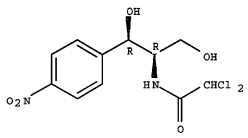
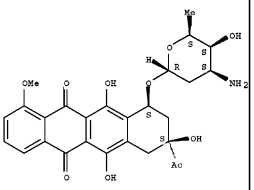
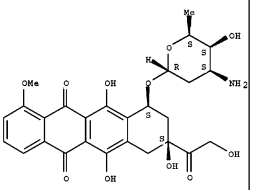
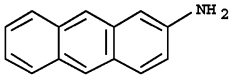
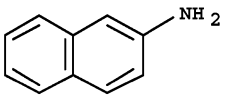
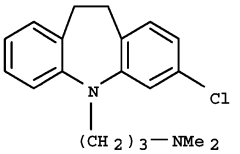
Table 1. Structure of the Substrate

ethidium bromide	chlorpromazine	acridine orange	proflavin	chloramphenicol
				
daunomycin	**adriamycin**	**2-anthramine**	**2-naphthylamine**	**clomipramine**
				

potentially damaging effects on nucleic acids and to demonstrate its use in drug and environmental monitoring. Advantages of the proposed technique include low cost and rapid and simple operation, with sensitivity comparable to that of the electrochemical method. The optical sensor was used for the rapid detection of polycyclic aromatic amines, antibiotics, and antitumor drugs (Table 1).

EXPERIMENTAL SECTION

Materials. The fluorescent dyes TP3 and SYBR Green I were obtained from Molecular Probes (Eugene, OR). Ethidium bromide (EtBr) was purchased from ICN Biomedicals Inc. (Irvine, CA). Low-molecular-weight DNA from salmon sperm and calf thymus DNA were obtained from Fluka (Stockholm, Sweden). Plasmid pBR322 and the *Hin*dIII restriction endonuclease were bought from Invitrogen Corporation (Carlsbad, CA). All other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). The glass end-to-end capillaries were purchased from Sarstedt AG & Co. (Numbrecht, Germany). The fluorescence system, QuantaMaster model C-60/2000, and the quartz fiber (060-8011) were purchased from Photon Technology International (Lawrenceville, NJ), including the light source (75-W xenon compact arc lamp), monochromators (model 101M) and detector (model 814). The analysis software was Felix Ver. 1.41. The glass imaging probe was obtained from Oriel Instruments (Stratford, CT). The fiber-optic R400-7 was purchased from Ocean Optics BV (Dunedin, FL).

Instrumentation. The setup for fiber fluorometry is illustrated in Figure 1.The optical fiber bundle was attached to the excitation monochromator to excite the sample from one end of the capillary. A lens system was used to collect the light emitted along the capillary and focus it onto the aperture of a second optical bundle attached to the emission monochromator. All slit widths in the system were set to 1 mm, corresponding to a band-pass of 4 nm.

Procedure. The binding assay procedure involves mixing different concentrations of DNA with intercalator dye solution and then reading the fluorescent intensity after incubation using a photomultiplier tube. The capillary tubes were washed with acetone and distilled water three times by applying sonication.

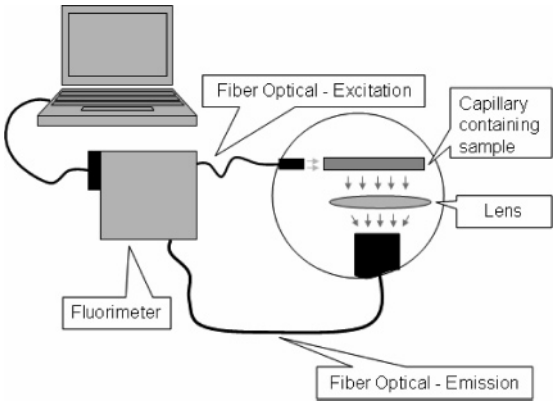


Figure 1. Setup of the fiber-optic fluorometer instrument.

The DNA solution was prepared as follow: 26 μL (0.25 μg/μL) of plasmid pBR322 solution was added to a centrifuge tube, followed by 1 μL of *Hin*dIII restriction endonuclease and 3 μL of REact 2 buffer. The reaction mixture was briefly vortexed and placed in a water bath (37 °C) for 1 h, and then the temperature was increased to 70 °C for 20 min to stop the reaction. The final solution containing 0.22 μg/μL pBR322 was stored at 4 °C. The TP3 stock solution was prepared by diluting the concentrated dye 200-fold with pure water to give a concentration of 5 μM and stored at −20 °C. The fluorometric assay was performed as follows. The TP3−DNA solution was prepared by mixing, in the following order: 25 μL of TP3, 63 μL of TE buffer (1 mM EDTA and 100 mM Tris-HCl, pH 8.0), and 2 μL of DNA solution. The mixture was vortexed immediately after the addition of DNA. The solution was incubated for several minutes at room temperature in the dark. After incubation, 10 μL of the competitors was added to the mixture and the resultant mixture stored in the dark. The final concentrations of the DNA and dye were 4.4 μg/mL and 1.25 μM, respectively. After 15-min incubation, the samples were transferred to capillary tubes (10−20 μL), which were then sealed at one end and mounted at the end of the fiber optic. The capillary tube was wiped clean with a tissue, and the fluorescence signal was read in the fluorometer in triplicate. The use of capillaries provides a simple, low-cost system requiring only small amounts of sample.

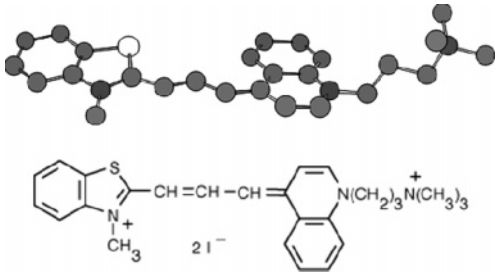


Figure 2. Structure of TP3.

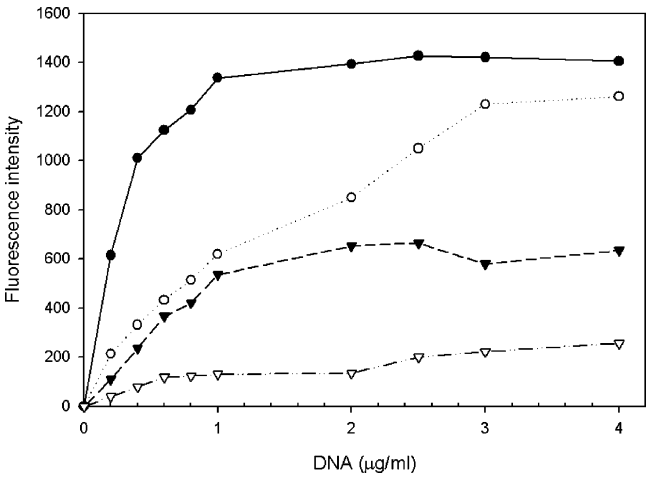


Figure 3. Comparison of fluorescence signals over a range of nucleic acid concentrations using different DNA−TP3 complexes: (●) digested plasmid pBR322, (○) plasmid pBR322, (▼) calf thymus DNA, and (▽) salmon sperm DNA.

To avoid the effects of background light, all measurements were performed in darkness.

RESULTS AND DISCUSSION

The structure of TP3 is shown in Figure 2. TP3 is a long-wavelength nucleic acid dye, which has maximal excitation and emission at 642 and 661 nm, respectively. The fluorescence intensity is proportional to the binding between the intercalator dye and the DNA.

Initially studies were performed to see how different types of DNA affected the fluorescence intensity. We compared DNA from different species, including salmon sperm chromosomal DNA (low molecule weight), calf thymus chromosomal DNA, plasmid DNA pBR322, and plasmid DNA pBR322 digested by the restriction endonuclease *Hin*dIII. As Figure 3 shows, the fluorescence intensity was greatly influenced by the choice of DNA. It was lower when the salmon sperm chromosome DNA and the calf thymus chromosome DNA were used as substrate. This may be due to the result of shearing during sonication in the production of DNA, giving a mixture of DNA of different lengths. For further investigations, we chose the well-defined nucleic acid material from plasmid DNA pBR322, which is one of the most commonly used *Echerichia. coli* cloning vectors. It is 4361 bp long and has a unique cleavage site for restriction endonuclease *Hin*dIII. From Figure 3 it can be seen that the higher the molecular weight of the DNA used, the faster the binding and the stronger the fluorescence intensity. This may be due to the longer nucleic acid molecule enhancing the fluorescence intensity by accumulating

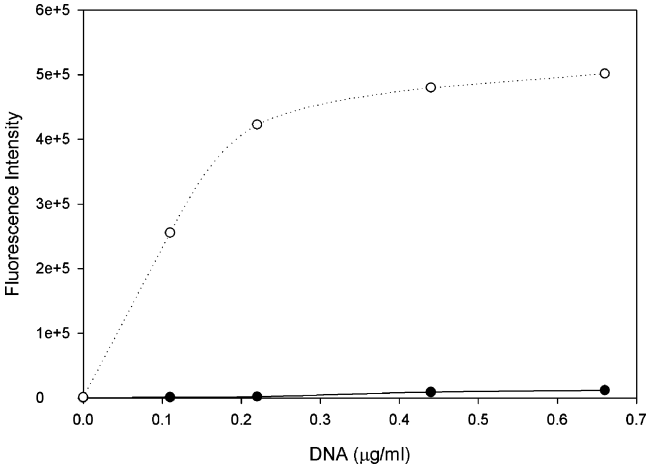


Figure 4. Comparison of fluorescence intensity using two different intercalate dyes binding to digested pBR322 plasmid DNA: (○) SYBR Green I and (●) TO-PRO-3.

the individual fluorescence signal produced by a DNA base pair. It is not surprising that the saturated fluorescence intensity caused by the digested plasmid DNA pBR322 was much higher and appeared faster than the others. This is due to the difference in the DNA configuration: the plasmid DNA is a circular piece of DNA in solution, and the supercoil configuration makes it difficult for the intercalator to bind to the double-helix structure. Nevertheless, after digestion by endonuclease *Hin*dIII, the plasmid pBR322 takes on a linear configuration, which allows the dye molecule to become intercalated into the DNA duplex, thus improving the ultimate binding.

The sensitivity of the assay strongly depends on the intercalating dye. We compared SYBR Green I, which is a dye specific to double-stranded DNA, widely used for DNA staining.[11,12] SYBR Green I is by far the best high-insensitivity reagent for nuclei acid staining.[8] As can be seen from the binding curve shown in Figure 4, the fluorescence intensity of SYBR Green I is significantly higher than that of TP3, but the competition assay indicated that TP3 had a higher potential to be displaced by a competitor; namely, it is more sensitive for fluorometric competition assays. This is because SYBR Green I has a much stronger binding affinity than TP3, which makes it difficult to be displaced by the competitor. The two positive charges and one intercalating unit afford TP3 a somewhat reduced affinity for nucleic acid binding, but a higher degree of displacement.[10]

The reaction time is crucial for the experiment, but since the incubation time of the TP3−DNA mixture has been described previously,[4] we focused here on the investigation of the competition time. The competition time for the competitive assay affected the fluorescence intensity, and 15 min was found to be optimal. Longer competition times showed no improvement in the reaction; on the contrary, there was a slight decrease in intensity that could be due to light bleaching. The buffer was also studied, and it was found that changing the pH within the range of 5.5−8.5 did not affect the decrease in fluorescence intensity (data not shown). On the other hand, changes in the ionic strength have previously been found to have a considerable influence.[4]

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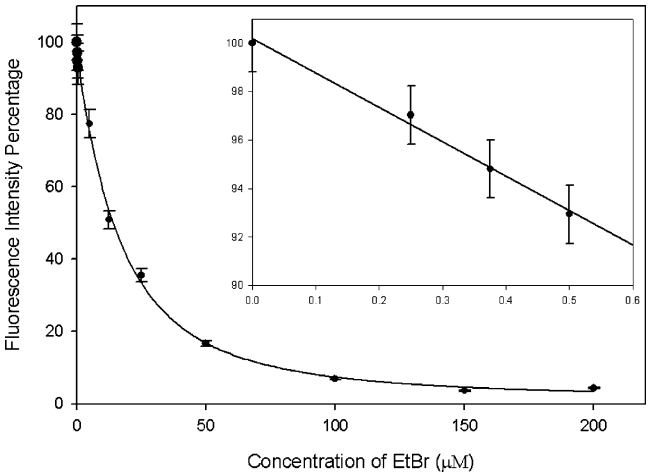

Figure 5. Calibration curve using ethidium bromide and TP3−DNA complex (digested plasmid pBR322).

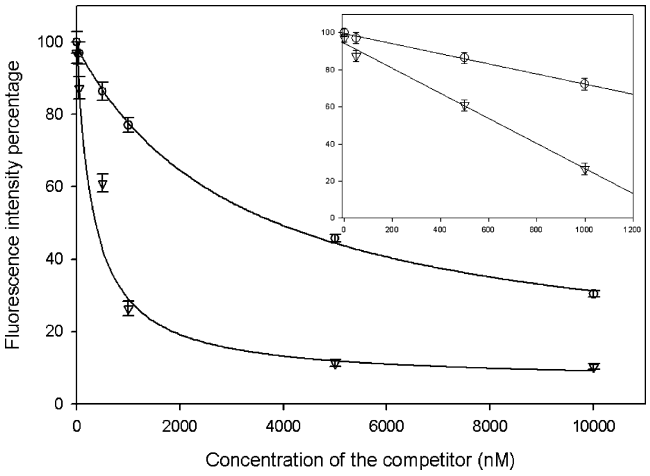

Figure 6. Competition curve for daunomycin (○) and adriamycin (▽) using TP3−DNA complex (digested plasmid pBR322).

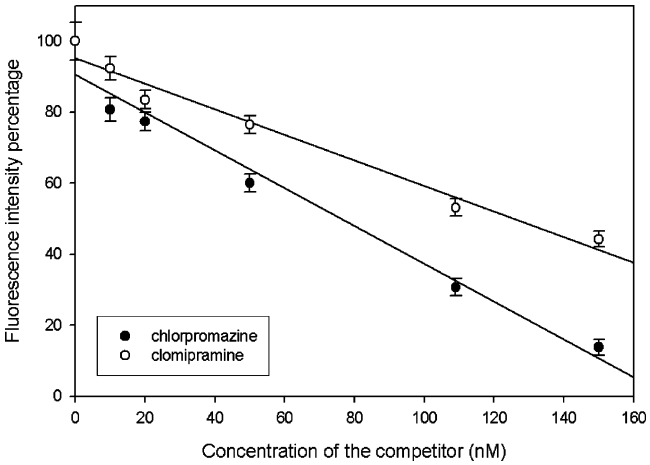

Figure 7. Competition curve for chlorpromazine (●) and clomipramine (○) using TP3−DNA complex (digested plasmid pBR322).

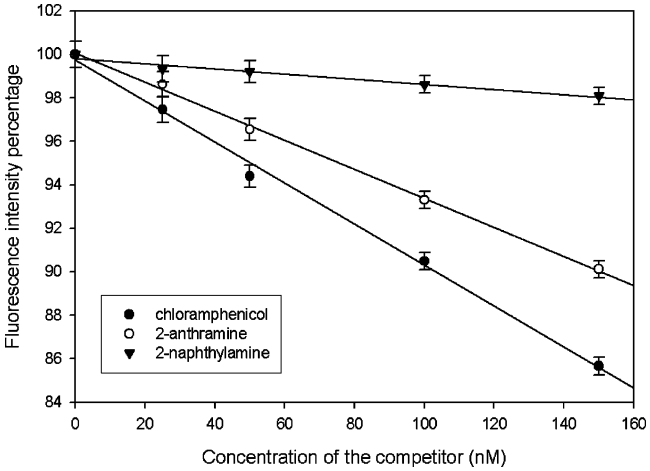

Figure 8. Competition curve for chloramphenicol (●), 2-anthramine (○), and 2-naphthylamine (▼) using TP3−DNA complex (digested plasmid pBR322).

To evaluate the sensitivity of the optimized system, we used the well-defined dye ethidium bromide as a reference intercalator (Figure 5). The assay had a linear range of 0.1−20 μM, and the detection limit was 100 nM with a relative standard deviation within ±5%. A series of experiments was performed using the optimized competition assay on several drugs (Figure 6). Adriamycin and daunomycin are clinically useful anthracycline antibiotics with a wide spectrum of chemotherapeutic applications.[13] They have been used against many cancer forms for decades.[14] Their antitumor activity has been attributed to the intercalation of the planar aromatic ring system between the DNA bases.[15] Some investigations of the interaction with DNA using electrochemical methods have been described previously.[6,7] We performed the competitive assay using a fiber optical device. The linear detection range for daunomycin was 50−1000 nM. Adriamycin could be measured up to 1000 nM with a steeper slope than for daunomycin, which means a higher sensitivity. The detection limit for adriamycin was somewhat lower than 50 nM. As in the electrochemical method, the potential for other applications of the proposed method is good. Chlorpromazine and clomipramine are tricyclic agents with both antidepressant and antipsychotic properties, and both have the ability to intercalate/interact with dsDNA, as shown in Figure 7. The improvement here over previous results[16] is due to the newly optimized system and the choice of a different DNA.

Chloramphenicol (CAP) is often used in the treatment of infections caused by bacteria; it has been shown to be harmful to humans as it can cause aplastic anemia, which can lead to leukemia.[17,18] The European Union (EU) has established safe maximum residue limits for substances authorized for use as veterinary drugs in food-producing animals (Council Regulation EEC 2377/90). Various analytical methods have been developed for the determination of CAP using gas chromatography, employing either electron capture or mass spectrometric detection as trimethylsilyl derivatives.[19,20] Gikas et al. have described a method of detecting the amount of chloramphenicol in seafood.[22] The high

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assay cost is, however, a disadvantage that must be overcome. Figure 8 shows the competition curve for chloramphenicol. The linear detection range is 10−150 nM, which makes this low-cost, easy-to-handle method a potential alternative for testing for chloramphenicol in seafood or other agricultural products. We also used DNA as a sensing element in the recognition and monitoring of toxic aromatic amines, such as 2-naphthylamine and 2-anthramine. As Figure 8 shows, different interaction effects appear due to the different structures. The decrease in fluorescence intensity caused by 2-naphthylamine proceeds slowly, while 2-anthramine causes a more obvious competitive effect. Within concentration of 100 nM, the effect is approximately linear. The same substances have been tested using an electrochemical DNA biosensor[22] with a detection limit and detection range comparable to those of our newly developed optical sensing method.

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CONCLUSIONS

The novel fluorescence-based optical biosensor described here is able to detect a wide range of substances with affinity for nucleic acids. The sensor is based on the decrease in the fluorescence intensity resulting from the formation of a complex between the sensing DNA sequence and an intercalating dye upon interaction with the analyte. TP3 was found to be a suitable dye with respect to fluorescence intensity and sensitivity to the presence of various analytes of interest. Moreover, for the primarily intended application as a sensor for broad-range environmental screening of earth and soil samples, the sensor has adequate sensitivity for the monitoring of antitumor drugs and certain water pollutants.

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