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Detection of DNA adducts of benzo[*a*]pyrene using immunoelectrophoresis with laser-induced fluorescence Analysis of A549 cells

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Abstract

Detection of benzo[*a*]pyrene diol epoxide (BPDE)-damaged DNA in a human lung carcinoma cell line (A549) has been performed using free zone affinity capillary electrophoresis with laser-induced fluorescence (LIF). Using BPDE as a model carcinogenic compound, the speed, sensitivity and specificity of this technique was demonstrated. Under free zone conditions, an antibody bound adduct was baseline-resolved from an unbound adduct in less than 2 min. The efficiencies of separation were in excess of $6\cdot10^5$ and $1\cdot10^6$ plates per meter for the antibody-bound and unbound adducts, respectively. Separation using a low ionic strength buffer permitted the use of a high electric field (830 V/cm) without the loss of resolving power. Using LIF detection, a concentration detection limit of roughly $3\cdot10^{-10}$ *M* was achieved for a 90-mer oligonuleotide containing a single BDPE. The use of formamide in the incubation buffer to enhance denaturing of DNA did not affect the stability of the complex between the antibody and the adducts. Using a fluorescently labeled BPDE-modified DNA adduct probe, a competitive assay was established to determine the levels of BPDE–DNA adducts in A549 cells.
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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute a large class of chemicals with widespread occurrence in the environment, to which humans are exposed [1–3]. Benzo[*a*]pyrene (B[*a*]P), which is one of the most common and extensively studied PAH, exhibits strong carcinogenic properties in experimental animals [4–6]. In vivo, B[*a*]P is converted to benzo[*a*]pyrene diol epoxide (BPDE), which covalently bond with DNA, primarily with guanine, initiating a critical step in cancer induction [7–11]. Bulky adducts in DNA represent a group of DNA damage whose repair involves a nucleotide excision repair pathway. DNA repair is an essential mechanism to protect against cancer. Because of the biological significance of DNA damage and repair, many techniques have been developed for the determination of DNA damage [12].

The most commonly used techniques for measuring DNA damage include ^{32}P-postlabeling assays [13–17], single cell gel electrophoresis (comet assay)

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[18–20], gas chromatography with mass spectrometry (GC–MS) detection [21–25] and immunoassays [26–35]. These assays each have their advantages and disadvantages with respect to sensitivity, specificity and background levels. The most sensitive method for detecting DNA damage is the radioactive ^{32}P-postlabeling method, which allows the detection of one adduct in 10^9 unmodified nucleotides in microgram amounts of DNA [13–17]. This technique requires working with hazardous radioactive material and time-consuming, multiple chromatographic separation procedures. In addition, the ^{32}P-postlabeling method in general does not provide information on adduct identity. The single cell gel electrophoresis, or comet assay, is very sensitive, but it is primarily for the analysis of DNA strand breaks [18–20]. GC–MS methods require enzymatic digestion and chemical derivatization of the DNA, leading to potential artifacts because the extensive DNA treatment procedures can introduce oxidative damage to the DNA. Electrochemical techniques are primarily for the detection of modified bases that are electrochemically active [36–38].

Development of DNA damage assays continues to be an active area of research. Recent advances in mass spectrometry have led to a number of reports making use of mass spectrometry detection with high-performance liquid chromatography (HPLC) [39–45], capillary electrophoresis (CE) [45–47] and capillary electrochromatography separation [48–50]. Various fluorescence techniques, such as fluorescence line narrowing, synchronous fluorescence and laser-induced fluorescence, have also been studied for DNA damage analysis [51–55]. Several techniques are based on the polymerase chain reaction (PCR) [56–61], which allow for the identification of DNA damage at specific locations in the genome. While these new developments are useful for measuring relatively high levels of DNA damage, a major analytical challenge remains to be the high sensitivity required for detecting low levels of DNA damage induced by clinically and environmentally relevant exposure to DNA damaging agents.

The objective of our research program is to develop specific assays for trace levels of DNA adducts. It makes use of a specific antibody to bind DNA adducts, capillary zone electrophoresis (CZE) for separation and post-column laser-induced fluorescence (LIF) for detection.

In this study, a synthetic BPDE–DNA adduct was used as a standard probe in a competitive assay to determine the levels of BPDE–DNA adducts in a human lung carcinoma cell line exposed to BPDE. A fluorescently labeled BPDE–DNA adduct standard and a BPDE-specific antibody were added to a sample containing an unknown amount of unlabeled BPDE–DNA adduct. The unlabeled BPDE–DNA adduct and the labeled BPDE–DNA adduct compete to form complexes with the antibody. CE separation of the bound and unbound adducts allows determination of the bound concentration, which in turn is related to the amount of BPDE–DNA adduct in the sample. In contrast to other methods of performing immunoassays, CE–LIF allows rapid analysis, excellent mass sensitivity and potential for automation. The popularity of this technique in immunoassays is well reflected in numerous reports, primarily for the determination of therapeutic drugs [62–67].

2. Experimental section

2.1. Preparation of BPDE–DNA adduct standard

Detailed protocols for the preparation and purification of a BPDE–DNA adduct standard are described elsewhere [68]. Briefly, BPDE powder {benzo[*a*]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (+/−) (anti)} was obtained from Midwest Research Institute (Kansas City, MO, USA. MRI 0477; lot CSL-98-775-17-16). The BPDE powder was dissolved in dimethyl sulfoxide (DMSO) to a stock solution of 3 m*M*. A 16-mer oligonucleotide, 5′-CCCATTATGCATAACC-3′, was treated with BPDE at a molar ratio of 1:5 (oligonucleotide:BPDE), using a protocol similar to that described by Cosman et al. [69]. The oligonucleotide was reconstituted in a buffer containing 20 m*M* phosphate–1.5% triethylamine at pH 11. To the oligonucleotide, a BPDE solution was added to a final concentration of 270 μ*M*. The final mixture was incubated in the dark at ambient temperature overnight with gentle shaking. Purification of the BPDE-modified oligonucleotide was carried out in two separate rounds of HPLC elution using a preparative column (Phenomenex, Torrance, CA, USA. Luna Su C_{18}(2); 250×10 mm, 5 μm particle size). In the first round, an isocratic elution using 70% methanol and

30% of 20 m*M* phosphate, pH 7 was used to purify the BPDE–oligonucleotide by separating the oligonucleotidess from the unreacted BPDE. The eluent containing the BPDE–oligonucleotide and the unmodified oligonucleotide was freeze dried and subjected to a second round of HPLC purification using a gradient elution of methanol–20 m*M* phosphate at pH 7. This purification step separates the BPDE–oligonucleotide from the unmodified oligonucleotide. The freshly purified BPDE–oligonucleotide was subjected to a standard kinase reaction to facilitate subsequent ligation to five other oligonucleotides to form a BPDE–DNA duplex of 90 base pairs (Fig. 1). The BPDE–DNA duplex was gel purified using a 7.5% native polyacrylamide gel, and subsequently subjected to UV and fluorescence scanning to measure DNA concentration as well as to confirm the presence of the BPDE moiety on the 90-mer.

2.2. Specific monoclonal antibodies

Monoclonal antibodies 8E11 and 5D11 were obtained from BD PharMingen (San Diego, CA, USA). Both antibodies were derived from BALB/c mice immunized with racemic anti-BPDE modified guanosine conjugated with bovine serum albumin [70,71].

2.3. Preparation of BPDE–DNA adducts from A549 cells

A human lung carcinoma cell line (A549) was incubated with BPDE to produce DNA adducts in genomic DNA. Briefly, the cell line was maintained in DMEM/F12 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum. The cells were seeded at 1×10^5 cells per plate and maintained at 95% humidity and 5% CO_2 for 20 h prior to the addition of BPDE. Treatment of BPDE was carried out in duplicate sets of A549 cells. Old culture media were removed from each culture plate and the cells were washed twice with phosphate-buffered saline (PBS). Media containing BPDE at various concentrations (9.4, 18.8, 37.5, 75, 150 and 300 μ*M* final concentration) were added accordingly to the designated plates. The cells were further incubated in the media containing BPDE for 2 h. The cells were then washed with PBS prior to the addition of DNAzol lysis reagent (Gibco BRL) to facilitate cell lysis. Subsequent steps involved a standard 99.9% ice cold ethanol precipitation and a

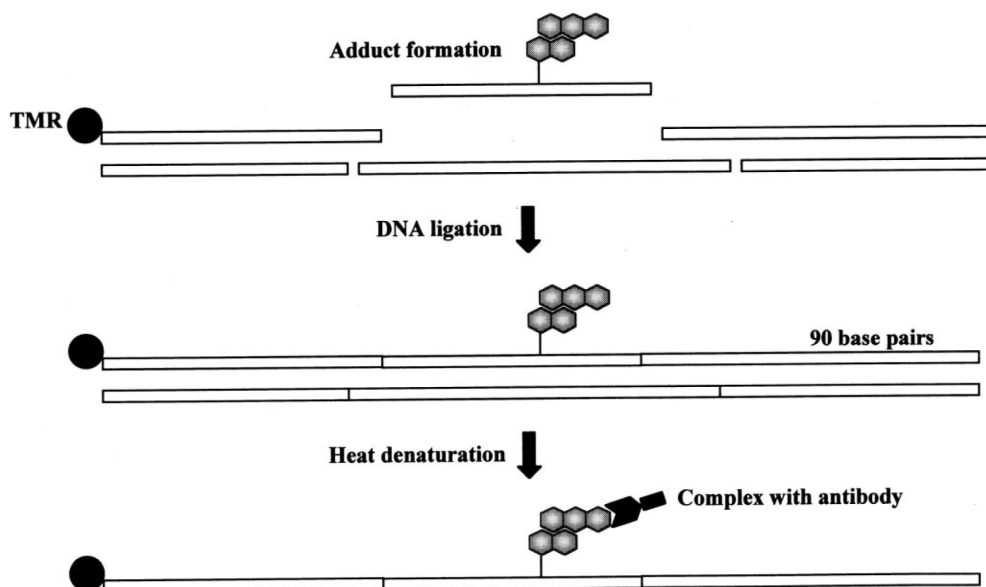


Fig. 1. Construction of a fluorescently-labeled BPDE–DNA standard. A 90 base-pair oligonucleotide duplex was constructed by ligating six smaller oligonucleotides. The strand containing the BPDE adduct was labeled at its 5′ end with tetramethylrhodamine (TMR). The double stranded oligonucleotide was denatured before reacting with anti-BPDE antibody to form immuno-complexes.

70% cold ethanol wash to purify the genomic DNA. The final DNA pellet was dissolved in distilled deionized water (ddH_2O) and the DNA concentration was measured at A_{260} using ddH_2O as a blank.

2.4. CE–LIF instrumentation

The instrument for capillary electrophoresis with laser induced fluorescence detection was built in-house and has been described in detail elsewhere [72–75]. It was equipped with a high-voltage power supply (Model CZE 1000R, Spellman, Plainview, NY, USA); a 543.5 nm green He–Ne laser (Melles Griot, Irvine, CA, USA) with a 5 mW maximum output. A Power Macintosh 7600/120 computer was used to control the power supply via a PCI-MIO-16XH-18 input/output board (National Instruments, Austin, TX, USA) and an interface box (I–V converter) that transferred output from the instrument to the computer. A sheath-flow cuvette of 0.9-mm thick walls, 200×200 μm square inner bore, 2 cm long (NSG Precision Cells, Famingdale, NY, USA) was used to hold a capillary at the detector end for post-column detection. The laser beam was focused by a 10× (N.A. 0.25) microscope objective (Melles Griot) into the sheath-flow cuvette, just below the capillary end. The fluorescence was collected at 90° from the direction of excitation by a 60× (N.A. 0.7) microscope objective (LWD-M Plan, Universe Kogaku, Japan). The transmitted light was spectrally filtered with a 580DF40 band-pass filter and detected by an R1477 photomultiplier tube (PMT) (Hamamatsu, Bridgewater, NJ, USA). The PMT signal was transferred to the interface box and digitized by the input/output board. Data was collected at a sampling rate of 10 Hz.

2.5. CE separation

Capillary electrophoresis of the sample was performed using a 29 cm×20 μm I.D.×150 μm O.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). Electrophoresis buffer was a Tris–glycine mixture containing 25 m*M* Tris and 192 m*M* glycine at pH 8.3. The injection end of the capillary was set at a positive polarity and the other end installed inside the sheath-flow cuvette was grounded. Sample introduction was performed by electrokinetic injection at 10 kV for 5–10 s unless otherwise indicated. Separation was performed with an electric field of 330–830 V/cm.

2.6. Immuno-complex of BPDE–DNA adducts

The incubation conditions were optimized for short reaction time and stable complex formation between the BPDE–DNA adduct and its antibody. The incubation was carried out at room temperature for 10 min in the dark. The incubation buffer was identical to the separation buffer except at half the ionic strength. The effect of buffer ionic strength on complex stability was studied by using the Tris–glycine buffer at various concentrations.

2.7. Competitive binding of BPDE–DNA adducts

Two oligonucleotides, a 16-mer and a 90-mer, were used as probes for competitive immunoassay. They each contained a single BPDE adduct in the middle and both were fluorescently labeled at a 5′ end with a tetramethylrhodamine (TMR). Another adduct standard carrying an identical BPDE–DNA adduct was prepared. This adduct standard is 16 bases in length and not fluorescently labeled. This BPDE–16-mer competes with the TMR-labeled BPDE–90-mer or TMR-labeled BPDE–16-mer to form complexes with the BPDE antibody. To determine the levels of BPDE–DNA adduct in the A549 cells exposed to BPDE, the purified genomic DNA from these cells was analyzed and the adducts in the DNA competed with the TMR-labeled BPDE–90-mer standard for binding with the antibody 8E11.

3. Results and discussion

3.1. Free solution mobility of DNA adducts

Under free zone electrophoretic conditions, DNA fragments are not separable when driven by electro-osmotic flow alone because of the similar mass-to-charge ratio between DNA fragments. The immunoassay presented here makes use of an antibody to specifically form a complex with DNA adducts so that the complex can be separated from the free DNA. As shown in Fig. 2a, the antibody-bound

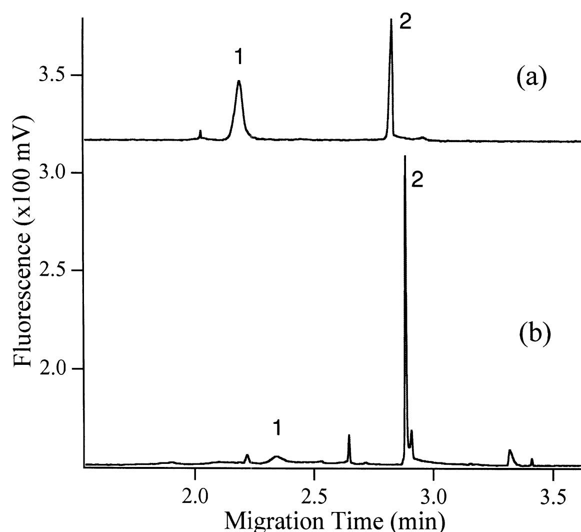


Fig. 2. Electropherograms showing the separation of BPDE–DNA adduct (peak 2) from its complex (peak 1) with antibody 8E11 (a) and 5D11 (b). The BPDE–90-mer was diluted to 7×10^{-9} *M* and reacted with 62.5 μg/ml antibody 8E11 or 5D11 in 10 μl 0.5× Tris–glycine buffer at pH 8.3. The reaction was carried out at ambient temperature in the dark for 10 min. The mixture was subjected to CE separation with LIF detection.

DNA adduct migrates out first and then the unbound DNA. This migration behavior can be expected from Eq. (1) [76]. This equation predicts the influence of the effective charge (Q), solution viscosity (η) and the radius of an analyte (r) on electrophoretic mobility (μ_{ep}). Both the antibody-bound and unbound DNA adducts are negatively charged. The direction of their electrophoretic mobility is opposite to that of the electroosmotic flow (EOF). The decrease in total charge-to-mass ratio after antibody binding decreases the mobility of the bound DNA adduct moving back to the injection end (positive polarity). The net result is a faster migration directed towards the detector end (direction of EOF).

$$\mu_{ep} = Q/6\pi\eta r \tag{1}$$

Comparing the two antibodies for their affinity to the BPDE–90-mer, antibody 8E11 (Fig. 2a) formed more complex than the antibody 5D11 did (Fig. 2b). This may reflect the fact that 8E11 was raised against BPDE mononucleotides and 5D11 was raised against BPDE modified calf thymus DNA. Thus, the 5D11 might be expected to have a higher affinity for long stretches of DNA.

3.2. Optimization of separation

To achieve separation efficiency and rapid analysis time while maintaining the stability of the antibody–DNA complex, we investigated the influence of capillary length, field strength and buffer ionic strength on the separation and detection of DNA adducts. Fig. 3 depicts the relationship between capillary length and stability of the antibody-bound DNA adducts during electrophoresis. As may be expected, the longer the capillary column, the more likely that the complexes may dissociate during electrophoresis. At a capillary length of 60 cm, the amount of detectable antibody-bound DNA adducts was reduced by approximately five-fold relative to an identical mixture separated on a 30-cm capillary. This reduction may be due to the instability of the

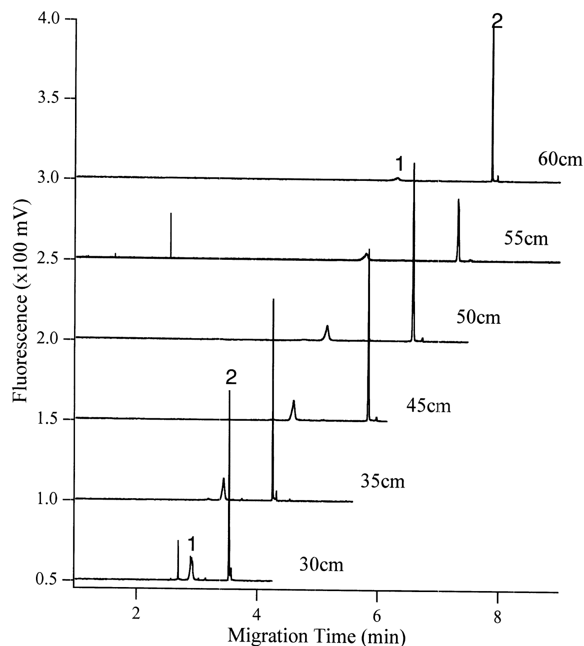


Fig. 3. Influence of the length of capillary column on the separation of a BPDE 90-mer oligonucleotide (peak 2) and antibody–oligonucleotide complex (peak 1). A capillary column was trimmed progressively from 60 to 30 cm. The field strength applied at each capillary length was adjusted accordingly to maintain a constant current across the capillary.

complexes during electrophoretic separation, or may be caused by adsorption of the complexes on the capillary wall. These problems could be avoided by using a shorter column to carry out the separation without losing resolution. In the absence of excess Joule heating, resolving power under free zone separation is independent of capillary length.

Separation at high field strength also helps to improve resolution. Under the influence of a high electric field, longitudinal diffusion of the antibody and the DNA adduct in the injected sample plug is minimized because of short separation time, thereby reducing dissociation of the complexes during electrophoresis. As shown in Fig. 4, at a field strength of approximately 830 V/cm (25 kV for 30-cm capillary), the antibody-bound and unbound DNA adducts were baseline resolved in less than 2 min, with a significant improvement in separation efficiency for the antibody-bound DNA adduct. Using Tris–glycine as the separation buffer, Joule heating was not excessive at this high field strength as the current generated was very low (~2.2 μA).

Buffer ionic strength is another parameter that may affect the stability of the complex and influence the interaction between the antibody and its binding site on the DNA adduct. In Fig. 5, the ionic strength of the separation buffer was varied from a stock buffer of 10× Tris–glycine (0.25 *M* Tris–1.92 *M* glycine). As the ionic strength of the separation buffer decreased, both resolution and plate count increased accordingly, with the optimum separation at 0.5× Tris–glycine (12.5 m*M* Tris–96 m*M* glycine). The plate count for the bound (peak 1) and unbound adducts (peak 2) using this buffer condition was calculated to be $6\cdot10^5$ and $1\cdot10^6$ plates per meter, respectively. When the buffer strength was decreased to 0.25×, a deterioration in separation was observed, presumably caused by a magnitude of electroosmotic flow that was too strong to permit the

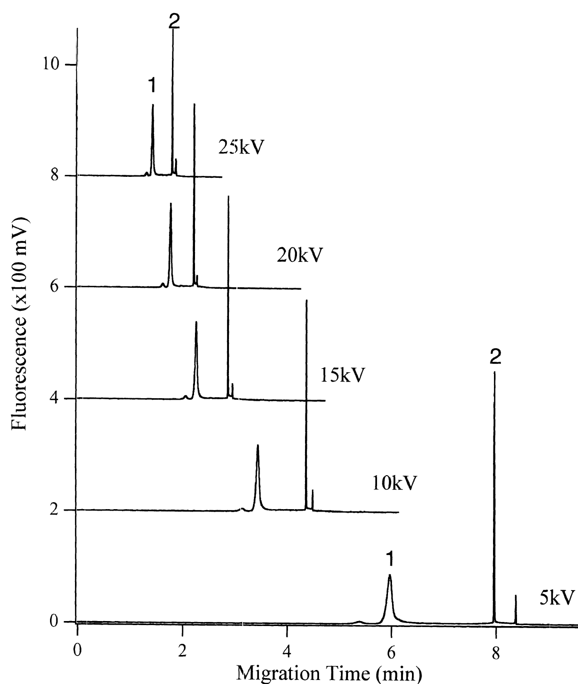


Fig. 4. Effect of field strength on the separation of antibody-bound (peak 1) and unbound DNA adduct (peak 2). Running voltage across a 30-cm capillary was increased progressively from 5 to 25 kV.

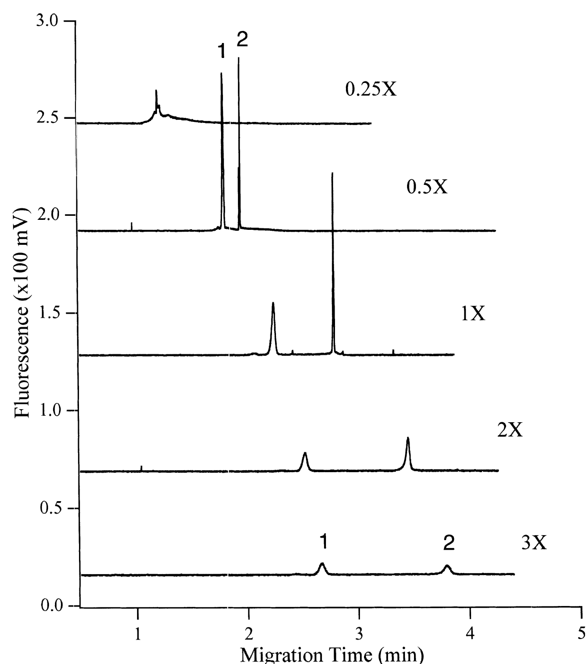


Fig. 5. Influence of ionic strength of the separation buffer on the separation of a BPDE–90-mer oligonucleotide (peak 2) and antibody–oligonucleotide complex (peak 1). A stock separation buffer of 10× Tris–glycine (0.25 *M* Tris–1.92 *M* glycine) was serially diluted to 3×, 2×, 1×, 0.5× and 0.25× with deionized water. Separation parameters are described in Section 2.

resolution of the bound and unbound BPDE–DNA adducts. As the ionic strength of the buffer decreased from 3 to 0.5×, there was a corresponding increase in the amount of complex detected, where the ratio of bound to unbound DNA adduct increased by approximately four-fold. This improvement could not be attributed to sample stacking. When the ionic strength of the separation buffer was reduced while the ionic strength of the sample was maintained constant, a decrease in sample stacking would be expected.

Incubation time and temperature were also investigated. We observed antibody binding to the DNA adduct at an incubation time as short as 1 min and temperature of incubation as low as 0°C. We found that an incubation between 5 and 10 min at ambient temperature was suitable for the formation of complex and for rapid sample analysis.

3.3. Denaturation of DNA

We compared the binding of the 8E11 antibody with double strand and single strand DNA. We found that the antibody bound better with the DNA adduct in single strand form. Thus, DNA was denatured prior to mixing with the antibody.

To ensure that the DNA remains in its denatured form, formamide was added to the incubation buffer to prevent the complementary DNA strands from being renatured during electrophoresis. Fig. 6 illustrates the effect of formamide on complex formation. We found that the presence of the denaturant (formamide) at a concentration up to 12.5% (v/v) did not affect the stability of the antibody-bound DNA adducts while improving the resolution of the bound and unbound BPDE–DNA adducts. Between 2.5 and 12.5% (v/v) formamide, the ratio of the bound to unbound adducts was relatively constant. As the concentration of formamide was increased to 82.5% (v/v), both the bound and unbound peaks became distorted. There was also a reduction in the ratio of the bound to unbound adducts. The reduction in the ratio of the bound to unbound adducts may indicate the onset of dissociation of the antibody from the DNA adduct. The higher peak intensity may be due to a larger injection volume, a result of the decrease in ionic strength of the sample buffer in a background of neutral formamide.

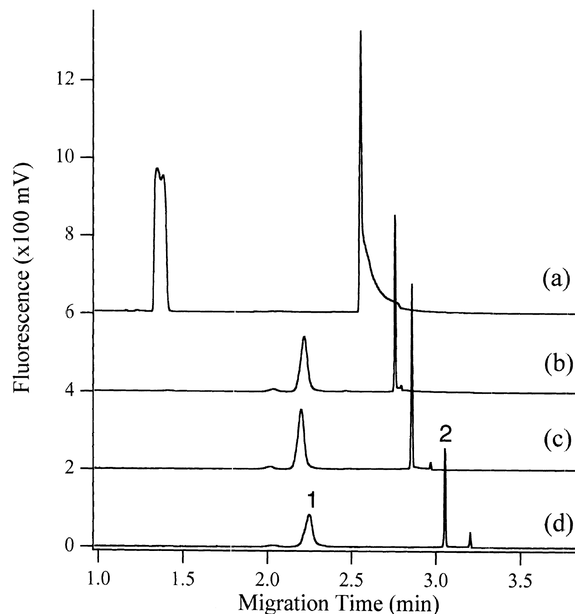


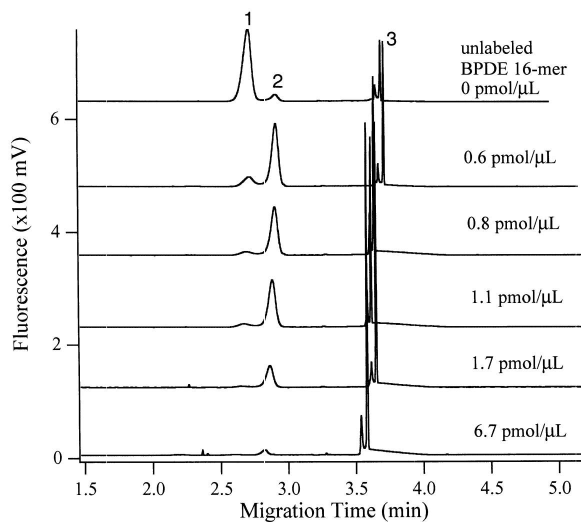
Fig. 6. Effect of formamide on the separation of a BPDE–90-mer oligonucleotide (peak 2) and antibody–oligonucleotide complex (peak 1). Incubation buffers containing formamide at concentrations (a) 82.5%, (b) 12.5%, (c) 5% and (d) 2.5% (v/v) were prepared for the reaction between the TMR-labeled BPDE–90-mer standard and the antibody 8E11. Conditions for incubation and CE analysis are described in Section 2.

3.4. Competitive assay

A competitive assay was performed using the TMR-labeled BPDE–16-mer as a probe and the unlabeled BPDE-16-mer as a competitor. Fig. 7a shows that the increase of BPDE–16-mer (unlabeled competitor) corresponds to the decrease of complexes (peaks 1 and 2) between the fluorescent BPDE–16-mer and the antibody. This is characteristic of competitive immunoassays.

The BPDE–90-mer that was fluorescently labeled with TMR was also used as a probe to demonstrate competitive immunoassay response with the unlabeled BPDE–16-mer (Fig. 7b). A similar competitive response was obtained as expected, suggesting that the antibody binds to the BPDE whether it is present in the 16-mer or the 90-mer oligonucleotides.

Both peaks 1 and 2 in Fig. 7 are due to complexes between the antibody and BPDE–DNA adducts. Because antibodies are bidentate, each antibody



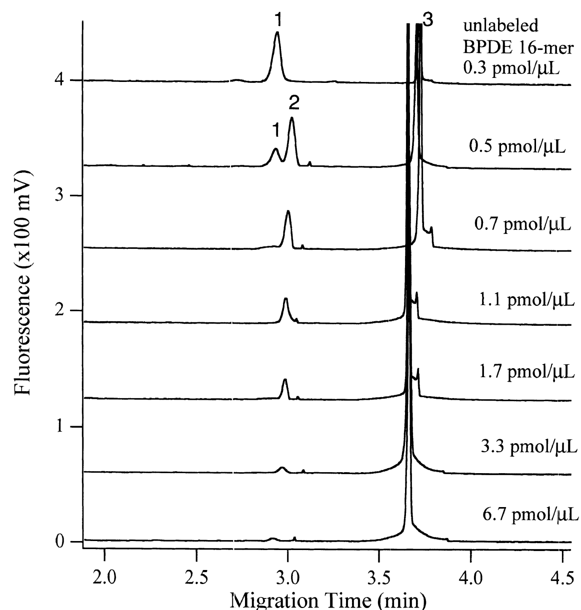


Fig. 7. Electropherograms showing competitive assay of unlabeled BPDE–16-mer using TMR-labeled BPDE–16-mer (a) and TMR-labeled BPDE–90-mer (b) as probes. Peaks 1 and 2 correspond to the 1:1 and 1:2 complexes between antibody 8E11 and the DNA adducts. Peak 3 corresponds to the unbound, TMR-labeled BPDE–DNA adducts.

molecule is able to bind with up to two antigen molecules. Peak 1 corresponds to the complex between one antibody and one DNA adduct. Peak 2 corresponds to the complex of one antibody with two DNA adduct molecules. The 1:1 and 1:2 complexes between the antibody and DNA adducts are well separated, demonstrating high resolution of the CE system. Detailed studies of the binding stoichiometry between the antibody and the DNA adducts will be described in a separate publication [77].

3.5. Determination of BPDE–DNA adducts in A549 cells

The competitive immunoassay was applied to the determination of BPDE adducts in A549 cells that were treated with various doses of BPDE (Fig. 8). The TMR-labeled BPDE–90-mer was used as the probe and the DNA from A549 cells was heat denatured. Fig. 8 shows that increasing amounts of

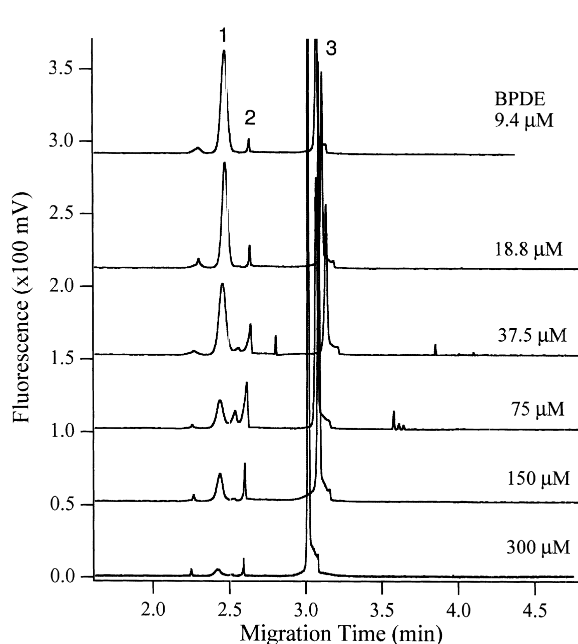


Fig. 8. Representative electropherograms showing competitive assays for BPDE–DNA adducts in A549 cells. TMR-labeled BPDE–90-mer was used as a probe (peak 3). The A549 cells were incubated with 9.4–300 μM BPDE for 2 h before the cellular DNA was extracted for adduct analysis. Peaks 1 and 2 correspond to the 1:1 and 1:2 complexes between antibody 8E11 and the DNA adducts.

BPDE–DNA adducts were formed as the cells were incubated with increasing concentrations of BPDE for 2 h. The BPDE–DNA adducts compete with the TMR labeled BPDE–90-mer probe for the antibody binding, resulting in the corresponding decrease of antibody complexes (peaks 1 and 2) of the fluorescent BPDE–90-mer. As in the case demonstrated in Fig. 7, peak 1 in Fig. 8 corresponds to the 1:1 complex between the antibody and the TMR-labeled BPDE–90-mer. Peak 2 in Fig. 8 is most likely the 1:2 complex of antibody with the TMR-labeled BPDE–90-mer and the DNA adducts from A549 cells.

Using the synthetic BPDE adduct 90-mer as a fluorescent probe and specific monoclonal antibodies to BPDE–DNA adducts, we demonstrated a rapid assay for BPDE-modified DNA in a human lung carcinoma cell line. This approach requires less than 4 min per separation and has excellent resolving power to separate the bound and unbound DNA adducts. The same approach may be extended to assays for other types of DNA damage.

Acknowledgements

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