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Evaluation of the Fiber-Optic Antibody-Based Fluoroimmunosensor for DNA Adducts in Human Placenta Samples

T. Vo-Dinh,[1] J. P. Alarie,[2] R. W. Johnson,[1] M. J. Sepaniak,[2] and R. M. Santella[3]

This paper presents the application of a fiber-optic antibody-based fluoroimmunosensor (FIS) for measuring DNA adducts of benzo[*a*]pyrene in human placenta samples. The FIS combines the excellent specificity of the antigen–antibody reaction, the high sensitivity of laser excitation, and the versatility of fiber-optic technology. The FIS was used to detect benzo[*a*]pyrene tetrol (BPT) after release from DNA by mild acid hydrolysis. These placenta samples contained one molecule of adduct per 10^7 base pairs. The limit of detection of the FIS for BPT is about 14 amol (14×10^{-18} mol).

Additional Keyphrases: *benzo[a]pyrene · fluoroimmunoassay · carcinogens*

Immunochemical techniques, which can offer excellent selectivity through antibody–antigen recognition, have revolutionized many aspects of chemical and biological sensor technologies. The high specificity and sensitivity of the techniques permit the measurement of many important compounds at trace amounts in complex biological samples. Radioimmunoassay (RIA), with radioactive labels, has been the most widely used immunoassay method. Radioimmunoassays have been applied to several fields, including pharmacology, clinical chemistry, forensic science, environmental monitoring, molecular epidemiology, and agricultural science. The usefulness of RIA, however, is limited by several shortcomings, including the cost of the instrumentation, the limited shelf life of radioisotopes, and the potential deleterious biological effects of radioactive materials. For these reasons, extensive research efforts have tried to develop simpler, more practical immunochemical techniques and instruments with sensitivity and selectivity comparable with that of RIA.

Recent advances in luminescence instrumentation, laser miniaturization, biotechnology, and fiber-optic research have provided opportunities for novel approaches to developing sensors for detecting human exposure to toxic chemicals and biological materials. (For a review of the development of fiber-optic chemical sensors, see references *1* and *2*, and the references therein.) In this study, we will present the operating principle of fiber-optic immunofluorescence biosensors. Examples of measurements will illustrate the application of a laser-based fluoroimmunosensor (FIS) developed to detect important biological compounds such as carcinogen metabolites and DNA adducts of carcinogens (*3*).[4] The FIS instrument derives its analytical selectivity through the specificity of antibody–antigen reactions (*3–8*). Antibodies are contained at the tip of the fiber-optic sensor for use in both in vitro and in vivo fluorescence assays. High sensitivity is provided by laser excitation and fluorometric detection. Further selectivity is achieved with phase-resolved detection (*9*).

An important polynuclear aromatic compound of great interest to toxicologists and cancer researchers is benzo[*a*]pyrene (BP), a ubiquitous byproduct of combustion of wood, fuel, and food materials (*10*) and a known animal carcinogen (*11*). Chemical processes, fossil energy production, coke-oven emissions, and diesel engine emissions are major outdoor sources of BP. Unvented kerosene space heaters, cigarette smoke, cooking fumes, and fireplaces can contribute to concentrations of BP indoors. BP is metabolically activated to electrophilic intermediates, which can bind covalently to DNA. A specific diol epoxide derivative of BP, *r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE), is the major carcinogenic metabolite involved in DNA binding. By converting the weakly fluorescent BPDE-DNA (by means of acid hydrolysis) to the highly fluorescent benzo[*a*]pyrene tetrol (BPT), the extent of BPDE-DNA adduct formation can be determined, which can provide evidence of an individual's exposure to BP.

Because DNA binding is the critical first step in carcinogenesis, analytical techniques capable of detecting carcinogenic DNA adducts have been developed to monitor human exposure to polynuclear aromatic compounds (*12*). In this study, we used for the first time FIS to analyze biological samples isolated from humans with environmental exposure to BP.

Materials and Methods

Instrumentation

Fiber-optic sensors may use either bifurcated or single-strand fibers (Figure 1). In the bifurcated design, separate fibers carry the excitation and emission radiation. A portable instrument with bifurcated fiber-optic waveguides has been described previously (*5*).

Here we used a single-fiber FIS instrument. Several

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[4] Nonstandard abbreviations: FIS, fluoroimmunosensor; BP, benzo[*a*]pyrene; BPDE, *r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; BPT, benzo[*a*]pyrene tetrol; and PBS, phosphate-buffered saline.

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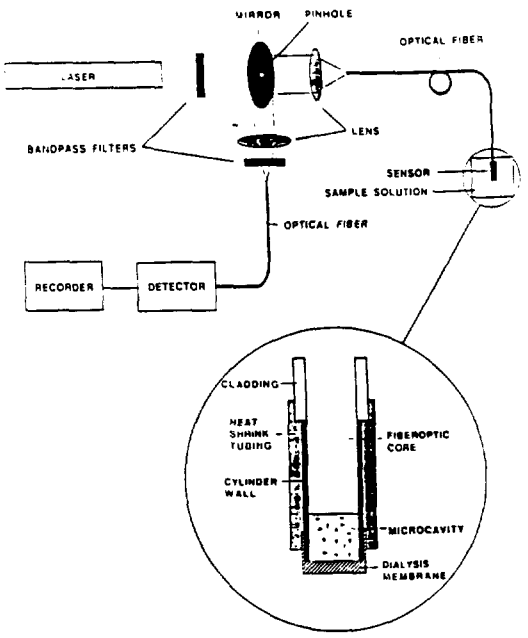

Fig. 1. Schematic diagram of the fiber-optic fluoroimmunosensor

FIS designs developed in our laboratories can have antibodies either covalently attached to the fiber-optic sensor tip (*3*) or contained in a microcavity (*6*). In this device, the sensor head consists of a membrane-enclosed microcavity, designed to contain the antibodies. A block diagram schematically illustrates the primary components of the FIS device used in this study (Figure 1). This FIS device is similar to that described in detail previously (*6*), but the optical fiber had a larger diameter (600 μm) than the previous one (200 μm). The larger-diameter optical fiber allowed easy focusing of the excitation radiation and better signal-collection efficiency. The excitation source was an Omnichrome Model 56X 10 mW He–Cd laser (Omnichrome, Inc., Chino, CA) operated at 325 nm. To eliminate the fluorescence from the antigen alone, we used a 40-nm bandpass filter with 40% transmission (Corion Corp., Holliston, MA) centered at 400 nm. A Model R760 photomultiplier tube (Hamamatsu Corp., Middlesex, NJ) biased at 800 V dc was used to detect the fluorescence emission. The photocurrent was processed with a picoammeter (Model 485; Keithley Instruments, Cleveland, OH) and displayed on a strip-chart recorder. A shutter, programmed to open for 1 s every 90 s, provided controlled-duration laser excitation and helped minimize photothermal and photochemical effects.

A schematic diagram of the membrane sensor head used in this study is also depicted in Figure 1. The membrane-enclosed sensor was constructed with 600/700-μm diameter core/cladding plastic-clad fused silica fiber (numerical aperture = 0.26) supplied by General Fiber Optics, Inc., Cedar Grove, NJ. Cellulose dialysis membrane (7-μm thick, molecular mass cutoff 10 000 Da; Diachema AG, Zurich, Switzerland) was stretched across the face of a piece of plastic heat-shrink tubing and positioned with a band of heat-shrink plastic. The tip was assembled so that it could slide on and off the fiber; when the tip is in place, a tight seal forms between the fiber jacket and the plastic tubing. About 2–3 mm of bare fiber core was exposed. The plastic heat-shrink tip was tapered, resulting in an inner diameter of roughly 600 μm. The distance between the membrane and the fiber face was adjusted to about 0.5 mm, yielding a sensor volume of ~140 nL.

Antibody Reagent

The monoclonal antibody 8E11 was developed from the spleen cells of a mouse immunized with BPDE-guanosine covalently coupled to bovine serum albumin (*13*). Cross-reactivity studies performed with a competitive enzyme immunoassay showed that the 8E11 antibody recognized DNA adducts and free BPT, BP-7,8 diol, and BP-9,10 diol, but not BP. The percentage of cross-reactivity when BPDE-I-DNA was the standard was 241% for BPDE deoxyguanosine and 140% for BPDE tetrols. K_a for the antibody–BPT complex formation in phosphate-buffered saline (PBS) was determined to be 10^8 L/mol.

Sample Preparation

Stored placenta DNA samples from a previous study (*14*) performed in collaboration with R. Everson, U.S. Environmental Protection Agency, Research Triangle Park, NC, were used. We hydrolyzed 333 μL of 1 g/L DNA preparation with 2.33 μL of concentrated hydrochloric acid at 90 °C for 90 min, then mixed 90 μL of the post-hydrolysis DNA sample with 90 μL of PBS (pH 7.4) and neutralized with NaOH, 1 mol/L. Total sample volume was 190 μL for each sample.

The control sample was lung DNA from an unexposed mouse, treated in the same manner as the experimental placenta samples.

Results and Discussion

Spectral Measurements

In this study, the FIS device was used to analyze biological samples isolated from humans. We selected human placenta samples for analysis for several reasons. One of our main goals in developing antibody-based sensors was to provide a sensitive and selective tool for detection and quantification of human exposure to environmental carcinogens. Our previous measurements demonstrated that the FIS can achieve attomole sensitivity (*6, 7*). These measurements, however, were performed with synthetic samples in PBS (pH ~7.3) containing BPT only or BPT with one or two other compounds. The human placenta samples had been found in previous studies to have low concentrations of BP–DNA adducts (*15*). We designed this study to evaluate the performance of the FIS device in these complex biological samples. Perhaps more traditional specimens such as blood components, blood cells, and biopsy specimens would be more appropriate samples in real life because of their ready availability. Because BPT might come from many sources (for example, smoking, cooking, and air pollution), determination of its origin in

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placenta samples requires further extensive studies and is beyond the scope of this work.

The difficulty of analyzing complex biological samples is illustrated in Figure 2, which shows the fluorescence spectra of a pure BPT sample and several placenta samples in response to excitation at 325 nm. This excitation wavelength was selected for spectral measurements because the FIS device uses the He–Cd laser 325-nm emission line. Figure 2*a* shows the fluorescence emission spectrum of BPT in PBS. The emission spectra were obtained by using a Perkin-Elmer (Norwalk, CT) Model 650 fluorescence spectrophotometer with a 2-nm spectral bandwidth. The fluorescence emission spectrum of BPT exhibits several resolved bands at 380, 400, and 422 nm and a wide shoulder at 445 nm. In contrast, the fluorescence emission spectra of placenta samples (Figures 2*b*, 2*c*, and 2*d*) are broad and featureless, with no discernible BPT peaks. The concentration of BPT (1 μmol/L) in Figure 2*a* was about three orders of magnitude greater than that expected in the placenta samples determined in previous analyses (*15*). BPT released from placenta DNA may not have been detected by fluorescence analysis with a conventional spectrofluorometer. In addition, the biological samples from humans with complex exposure may contain multiple adducts, and the emission from BPT may seriously overlap emission from other fluorescing compounds. For comparison purposes, the fluorescence spectrum of a control background, a control DNA sample from an unexposed laboratory mouse, is shown (Figure 2*e*).

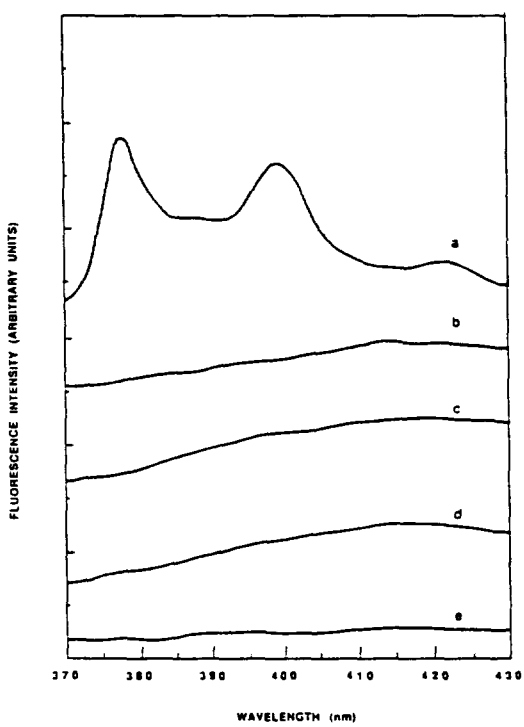

Fig. 2. Fluorescence spectra of BPT and biological samples: (*a*) solution of pure BPT in PBS, (*b*) human placenta sample 1, (*c*) human placenta sample 2, (*d*) human placenta sample 3, (*e*) control DNA sample

Real-Time Response

Figure 3 depicts the temporal response of the FIS. The membrane sensor was filled with 1.9 μmol/L anti-BPT antibody solution and placed in 1 mL of 5.9 nmol/L BPT solution. The antibody–BPT fluorescence emission was monitored for 20 min with the previously described shutter system. For blank measurements we filled different sensors with either protein-free PBS or non-BPT-specific rabbit IgG (2 μmol/L). Figure 3 also shows the FIS temporal response for placenta sample no. P116. The increase of the sensor signal is similar to that of the BPT sample; the slight difference in the temporal response could be due to a difference in the nature of sample media in the two experiments and to concentration differences. The BPT concentration of sample no. P116 was determined to be 4.85 nmol/L.

Quantitative Measurements

Measurements with the FIS can be performed either sequentially or in steps. Sequential measurements were performed by filling the membrane-sensor tip with antibody solution for each sample. After each measurement, the sensor head was refilled with fresh antibody. Each FIS was incubated in a 1-mL antigen solution for 15 min and rinsed in PBS solution for 5 min. During this period, BPT molecules diffused across the membrane into the sensor tip and were bound to the antibodies. When the sensor was rinsed in PBS, unbound antigens and interfering substances were dialyzed out of the sensing tip. Antibody-bound material remained, confined by the membrane to the fiber's viewing region. Signal was obtained either from the slope of the 15-min signal rise or from the difference between pre- and post-incubation signals in blank PBS solutions. Signal-to-noise values were calculated from the peak-to-peak noise of blank PBS solutions.

Because a relatively large amount of antibody remains unbound during these nonequilibrium dialysis measurements, stepwise measurements could also be

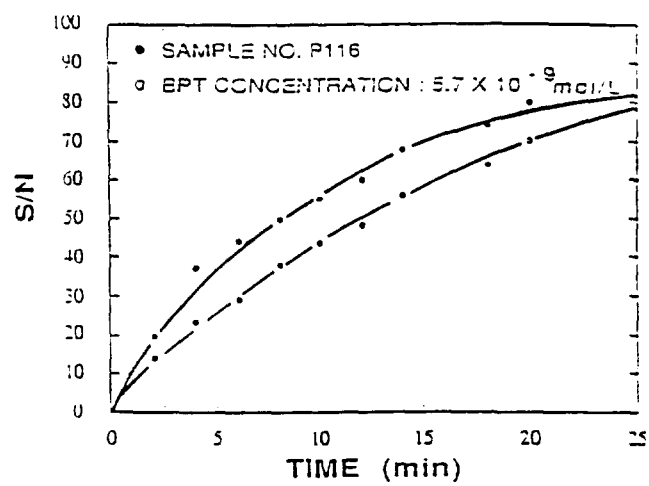

Fig. 3. Temporal response of the FIS for BPT in PBS (C) and in placenta samples (●)
S/N, signal-to-noise ratio

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performed with the FIS, i.e., loading the sensor tip with antibody and performing 15-min incubations. Rinsing the sensor in PBS marked the endpoint of the measurement and the new baseline for the next analysis. Both the slope and the signal difference were used to obtain data. Sensor response was independent of the measurement sequence of antigen solutions: signals obtained going from high to low concentrations were the same as those obtained going from low to high concentrations.

Figure 4 shows the calibration data obtained with a membrane-enclosed sensor. Antigen solutions containing BPT were prepared in PBS, at concentrations between 1.0 and 10 nmol/L. The sensor was filled with monoclonal anti-BPT antibody, 1.9 μmol/L, for these measurements. The results indicate that the measurements performed with BPT in PBS and in placenta samples were in the linear range of the calibration curve.

After calibration, we measured BPT in placenta samples by the stepwise procedure. Incubation of the sample for 15 min was followed by a 5-min incubation in PBS to remove interferents. For these experiments, each probe tip was used for only two measurements and was then replaced by a new tip. The concentrations of BPT in placenta samples were determined by comparing the background-subtracted intensities with those obtained in calibration measurements. The amounts of BPT detected in several placenta samples are summarized in Table 1, which also gives the amount of DNA adduct formed in these samples; these data were derived by determining the number of DNA base pairs in the original samples by using ultraviolet absorption spectrometry. The number of base pairs was determined from the DNA concentration after hydrolysis (3.3 g/L). Only 90 μL of the total sample was used, yielding a total of 0.3 mg of DNA. Approximating the formula weight of a single nucleotide as 327 g/mol yields 0.92 μmol of DNA in the 0.3-mg DNA sample. To calculate the number of base pairs, we multiplied the micromoles of DNA by Avogadro's number and divided by 2 (two base pairs per molecule), thus yielding the total of 2.7×10^{17} base pairs.

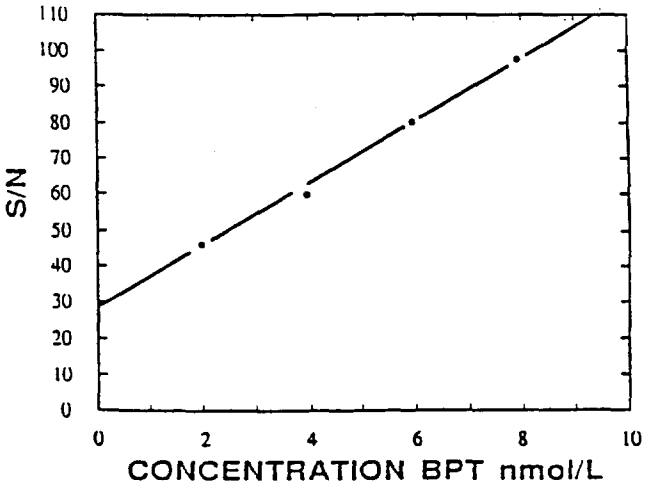

Fig. 4. Biosensor calibration curve for BPT in PBS
S/N, signal-to-noise ratio

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Table 1. FIS Measurements of BPT in Human Placenta Samples by the FIS Device

Sample	BPT concn, nmol/L	BP adducts, no. per 10^7 base pairs
P158	1.7	7
P251	3.2	13
P116	2.3	9
P112	2.6	11

Sample calculations (for sample P158): No. of BP adducts/base pair = $(1.7 \times 10^{-9}$ mol/L) $(190 \times 10^{-6}$ L) $(6.02 \times 10^{23}$ molecules/mol) = 1.9×10^{11} molecules. 1.9×10^{11} molecules/2.7×10^{17} base pairs = 7×10^{-7}.

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