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Antibody-Based Fiberoptics Biosensor for the Carcinogen Benzo(a)pyrene

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A new antibody-based fiberoptics biosensor was used to detect the important carcinogen benzo(a)pyrene (BaP). The fiberoptics sensor utilizes anti-BaP antibodies covalently bound to its tip. A helium cadmium laser was used as the excitation source to induce fluorescence from BaP conjugated to the bound anti-BaP antibodies. The fiberoptics device can detect 1 femtomole of BaP in a 5- μ L sample drop.

Index Headings: Fiberoptics; Sensor; Antibody; Immunofluorescence; Benzo(a)pyrene; Carcinogenic compound.

INTRODUCTION

For the past few years, biosensor technology has been at the forefront of analytical instrumentation research. The integration of biological systems and advanced optical sensor technology promises to open new horizons in medical, clinical, and environmental monitoring applications. In the area of human health protection against environmental pollutants, such as potentially carcinogenic polynuclear aromatic (PNA) compounds, there is a strong need for sensitive and selective monitoring instrumentation to analyze complex biological samples. The PNA compounds, which are produced in occupational and residential activities as a result of incomplete combustion of organic matter, are particularly important pollutants since many of them are carcinogenic.¹ With the advent of immunochemical techniques, which offer the capability of antibody-antigen recognition of trace amounts of specific antigens, many traditional aspects of chemical and biological monitoring technologies are experiencing dramatic changes. Radioimmunoassays (RIA) utilize radio-active labels² and comprise the most widely used immunoassay methods. The usefulness of RIA, however, is limited by several shortcomings, including the cost of instrumentation, the limited shelf life of radioisotopes, and the potential biological effects inherent to radioactive materials. For these reasons, there are extensive research efforts aimed at developing sim-

pler, more practical, but equally sensitive and selective analytical instrumentation.

The use of fiberoptics-based sensors has recently been reviewed.^{3,4} For the past few years our laboratories have been involved in the development of various fiberoptics-based monitors.⁵⁻⁹ In this study an antibody-based fiberoptics sensor using laser-induced fluorescence for benzo(a)pyrene (BaP) is described. Benzo(a)pyrene was selected as the model PNA compound because this important compound is found in many industrial and residential environments (chemical, petroleum, coke oven, and synfuel industries; wood-burning fireplace and cigarette smoke) and because it is known to be carcinogenic.¹ Recent advances in luminescence instrumentation, laser miniaturization, biotechnology, and fiberoptics research have allowed us to pursue the development of sensors which measure environmental and human exposure to toxic chemical and biological materials. Antibodies to BaP were produced by polyclonal techniques and were covalently attached to a fiberoptics sensing probe of a fluoroimmuno-sensor (FIS). A helium-cadmium laser was used as the excitation source. Results illustrating the feasibility and analytical capabilities for BaP detection at femtomole levels are presented. The potential utility of the FIS in cancer research, environmental monitoring, and health protection is indicated.

EXPERIMENTAL

Instrumentation and Procedure. A schematic diagram of the operating principle of the FIS apparatus is shown in Fig. 1. A detailed description of the FIS instrument development is given elsewhere.¹⁰ Only the main features of the device are described here. The excitation source was a helium cadmium laser (Omnichrome, Model 3112) operated at 325 nm. The laser radiation was directed through an optical beamsplitter and focused onto the incident end of an optical fiber (excitation path). A 50-mm-focal-length $f/2$ lens was used to focus the laser radiation onto the end of a 600- μ m-core-diameter, multi-mode, fused-silica optical fiber (Math Associates Inc., Model QSF-600). Previously developed fiber sensors are based on evanescent wave coupling of light out of a relatively short bare fiber into the sample, and vice versa.¹¹

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FIG. 1. Operating principle of the fiberoptics antibody-based biosensor. The instrument consists of an optical fiber having antibodies immobilized at the sensor tip; excitation radiation from a helium cadmium laser (325 nm) is sent through a beamsplitter onto the incidence end of the optical fiber. The laser radiation is transmitted inside the fiber onto the sensor tip, where it excites the analyte molecules [benzo(a)pyrene] bound to the antibodies. The excited antigen fluorescence is collected and retransmitted back to the incidence end of the fiber, directed by the beamsplitter onto the entrance slit of a monochromator, and recorded by a photomultiplier. The intensity of the fluorescence signal measured is proportional to the amount of antigen bound to the sensor tip.

The FIS device developed here utilized the back-scattering of the light emitted at the remote sensor probe. A single fiber was used to transmit the excitation radiation into the sample and collect the fluorescence emission. An optical fiber micropositioner was used to provide fine adjustments of the fiber's incident end. This allowed us to optimize transmission of the focused laser beam onto the sample. The laser radiation reached the sensor probe and excited the BaP bound to the antibodies immobilized at the fiberoptics probe. The BaP fluorescence was isolated by a bandpass filter and monochromator (Instruments SA, Model H-10) with a 8-nm bandpass and then transmitted back through the beamsplitter to the detector (emission path). The fluorescence was detected with an RCA IP28B photomultiplier tube operated at 800 V. The photocurrent was processed with the use of a Keithley (Model 485) picoammeter. Data were stored in the memory of the picoammeter and recorded with a strip-chart recorder.

In the design of the biosensor for BaP, antibodies directed against BaP must be developed. A substance capable of inducing an antibody response in the immune system is called "immunogenic." To be immunogenic, a substance must meet certain requirements in molecular size and complexity. Proteins with molecular weights of >5000 – $10,000$ are generally immunogenic, whereas smaller compounds such as BaP molecules are usually not immunogenic. In order to produce anti-BaP antibodies, one couples BaP molecules to a carrier protein, then injects them into laboratory animals in order to elicit the animals' immune response.

We produced antibodies directed against BaP by coupling BaP to bovine serum albumin (BSA). Benzo(a)pyrene-6-isocyanate was prepared from BaP by a three-step synthesis, following an established proce-

FIG. 2. Procedures for immunofluorescence measurement of benzo(a)pyrene. In this study the measurement of benzo(a)pyrene bound to the fiberoptics tip involved a three-step procedure: (1) The fiberoptics sensor tip was immersed into a 5- μ L drop of sample solution containing BaP; during the incubation time, set at 10 min, the BaP molecules, which diffused towards the sensor tip, were conjugated to the antibodies immobilized on the sensor tip. (2) Following incubation, the sensor tip was removed from the sample and rinsed with a PBS solution; this operation took about 10 s. (3) To conduct the measurement, we directed laser excitation radiation to the sensor tip by opening a shutter; the fluorescence from the BaP molecules excited by the laser radiation was measured for a few seconds.

dures.^{12,13} The BaP-isocyanate was reacted with BSA to produce the BaP-protein conjugate, which was used for animal immunization, following procedures previously described by Griffin *et al.*¹⁴ New Zealand white rabbits and Fisher rats were immunized by subcutaneous or intramuscular injection with BaP-BSA in Freund's Complete Adjuvant. Succeeding doses of BaP-BSA (0.1–0.15 mg/kg of body weight) in Freund's Incomplete Adjuvant were subsequently administered by subcutaneous injection at time intervals of 2 to 6 weeks. Serum from immunized animals was removed and tested for BaP-antibodies with the use of several techniques, including immunodiffusion in agar (Ouchterlony technique), passive hemagglutination, enzyme-linked immunosorbent assay (ELISA), and radioimmunoassay. The immunoglobulin G (IgG) fraction from immunized rabbits was isolated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and DEAE-cellulose chromatography.¹⁴

The experimental procedures used to prepare optical fibers and samples for fluorescence and radiolabeled BaP measurements were as follows: quartz fibers were stripped of their cladding for a length of 7 to 8 mm; bare fibers were then derivatized with 3-glycidoxypolytrimethoxysilane (GOPS), with the use of previously described procedures.¹⁰ Following oxidation with periodic acid, the fibers were incubated for 36 to 48 h in solutions containing 2 mg/mL of rabbit IgG (for the control fibers) or 2 mg/mL crude IgG fraction from sera of rabbits immunized with BaP-BSA (for the BaP-antibody fibers). Phosphate buffered saline (PBS) was used as the diluent for the IgG preparations. After covalent linking of the IgG protein to the fibers, the final step was reduction with sodium borohydride (NaBH_4). The fibers were then rinsed with PBS and were ready for use.

Chemicals and Reagents. Benzo(a)pyrene and 3-glycidoxypolytrimethoxysilane were purchased from Aldrich Chemical Co.; bovine serum albumin and phosphate buffered saline were purchased from Sigma

FIG. 3. Time response of the fiberoptics probe. In this experiment, a fiberoptics probe with immobilized BaP-antibodies was immersed into a solution of BaP (2×10^{-7} M, in PBS + 10% ethanol). The fluorescence measurements of BaP were performed by the experimental procedures described in the caption for Fig. 2. The fluorescence intensities were recorded at various time intervals. The time response curve shows that the intensity reaches half of its maximum value after 10 min.

Chemical Co. Multimode, fused-silica optical fibers were obtained from Math Associates.

RESULTS AND DISCUSSION

Antibody-based sensors such as the FIS can be used to perform various types of immunoassays, depending on the type of antigens. For fluorescing antigens, direct *in situ* measurements of the analytes bound to the antibodies on the sensor probe can be performed. In this study the FIS detection scheme for BaP is depicted schematically in Fig. 2. The sensor probe having immobilized antibodies against BaP is placed in a solution of BaP. Following incubation, the probe is removed from the solution, rinsed with a PBS solution, and directly analyzed. Since BaP is a fluorescent compound, there is no need to use fluorescent-tagged labels in the assay. The laser radiation transmitted along the optical fiber reaches the sensing probe and excites the BaP molecules that have conjugated with the anti-BaP antibodies covalently bound to the sensor. Continuation of the incubation procedure leads to a steady increase in fluorescence signal as more and more BaP molecules reach the bound antibodies.

The chemical, physical, and optical characteristics of the optical fibers are important factors in determining FIS capabilities. The fiber tip surface area can be increased by etching procedures. Quartz tends not to be

TABLE I. Determination of amount of ^3H -benzo(a)pyrene eluted from the fiberoptics sensor.

Elution scheme	Amounts of ^3H -BaP eluted (fmole)	
	From a sensor with BaP-antibodies	From a sensor with IgG-antibodies (control sensor)
(A) One-step procedure using 6-M KSCN chaotropic reagent	24	7
(B) Two-step procedure using		
(1) 6-M KSCN chaotropic reagent		
(2) 6-M KSCN + 10% dimethylsulfoxide	950	600

as effectively etched as glass. The material of the optical fibers also determines the usable spectral range. Fused-silica optical fibers permit measurements in the ultraviolet down to 220 nm, but are relatively expensive. Glass is less costly, but is only appropriate for measurement in the visible. Plastic is the least costly and most durable of materials, but these fibers are restricted to spectral ranges above 450 nm and are therefore not suitable for UV excitation. In this study the 325-nm emission line of a helium-cadmium laser was used for fluorescence excitation. Therefore, quartz optical fibers had to be used in this instrument.

Figure 3 shows the temporal response of the FIS signal following incubation in a BaP solution. The plateauing of the sensitivity curve after 1 h may be due to the saturation of the antibodies by the BaP molecules and indicated that steady-state conditions were reached. The results in this figure were obtained with a (2×10^{-7} M) solution of BaP in phosphate buffer/1% ethanol. After the first 10 min, the signal reached 50% of its maximum value. During the next 50 min, the fluorescence signals increased only by another 50% of the maximum value.

Experiments were conducted to compare the binding characteristics of the BaP sensor with those of a control sensor coated with rabbit IgG. The sensor coated with IgG was used to investigate the nonspecific adsorption of BaP on protein molecules. In these experiments, BaP sensors and IgG sensors, prepared by the procedures described in the experimental section, were incubated for 90 min at 37°C in 750- μL 2×10^{-7} M solutions of ^3H -BaP in the phosphate buffer/1% ethanol. Following incubation, the FIS probes coated with rabbit IgG and BaP-antibody were thoroughly washed with antibody disruptive (chaotropic) reagents in an attempt to elute the antibody-bound ^3H -BaP. Two different elution procedures were investigated. Table I summarizes the results of the elution experiments using liquid scintillation measurements for the BaP sensor and the control fiber. In the first procedure, only one elution solution, a 6-M potassium thiocyanate (KSCN) chaotropic reagent adjusted to pH 3.0, was used. With the BaP sensor, 24 femtomoles were recovered, whereas only 7 femtomoles of ^3H -BaP were recovered with the control IgG fiber. This experiment demonstrated that more ^3H -BaP could be eluted from the BaP sensor than from the control fiber.

FIG. 4. Performance of the fiberoptics antibody-based sensor at different concentrations. A fiberoptics sensor was used to detect fluorescence of various BaP amounts in 5- μ L sample drops.

In the second experiment, a two-step elution procedure was performed with the use of (1) 6-M KSCN, and (2) 6-M KSCN containing 10% dimethylsulfoxide (DMSO). Table I shows that, in the two-step process, 950 femtomoles of ^3H -BaP were eluted from the BaP sensor, whereas only 600 femtomoles of BaP were eluted from the control fiber. The results of the two experiments were not directly comparable, since different batches of fibers and quite different elution procedures were used. In both experiments, the results indicate that more BaP molecules were eluted from the BaP sensor than from the control fiber. This in turn suggests that the BaP sensor bound more BaP than did the control sensor. The data also indicate that the fiber sensors prepared with polyclonal antibody against BaP exhibited some degree of nonspecific binding. Since further improvements in the analytical utility of the fiber sensors can be achieved by increasing the avidity and specificity of antibodies, we are currently developing procedures to produce monoclonal BaP antibodies.

The response of the FIS device to various concentrations of BaP is illustrated in Fig. 4. The measurements were conducted with only 5- μ L samples. The FIS probes were incubated for 10 min in the BaP sample drops. Following incubation, the FIS was removed from the sample drop, rinsed with PBS, and placed in a PBS measurement solution. We then measured the fluorescence signal by excitation, using laser radiation and recording the emission signal. Each measurement took approximately 2 min, following the incubation process. An increase in the FIS signal was observed with an increase in the BaP amount in the samples. The results indicated that the FIS limit of detection was approximately 1 femtomole (10^{-15} mole) of BaP in a 5- μ L drop. This is based

on the observation of a signal-to-noise ratio of 2 for a 10-min incubation period. It is expected that longer incubation times would lower the detection limits. It is interesting to note that limits of detection for a bare fiber (no bound antibodies) are higher ($\sim 8 \times 10^{-10}$ mole) than those for the FIS. This illustrates, in part, the capability of FIS to concentrate samples at the sensing tip.

CONCLUSIONS

In this work we have successfully demonstrated the usefulness of an antibody-based fiberoptics sensor for the detection of the carcinogen benzo(a)pyrene. The excellent sensitivity of this device (femtomole limits of detection) illustrates that it has considerable potential to perform trace analyses of chemical and biological samples in complex matrices. Measurements are simple and rapid (~ 12 min), and the technique is applicable to other compounds, provided appropriate antibodies are used. Several parameters, however, must be further investigated and optimized before such devices can find utilization at their full potential. These factors include minimizing antibody cross reactivity, extending sensor stability and shelf life, and improving immobilization reproducibility. These areas are currently under investigation, and it is our opinion that fiberoptics-based FIS can be useful in a wide variety of biochemical and clinical analyses, including the assessment of an individual's exposure to chemical carcinogens, responses to drug therapy, and characterization of naturally occurring biologically active substances.

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