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

T. VO-DINH,* B. J. TROMBERG,+ G. D. GRIFFIN, K. R. AMBROSE, M. J. SEPANIAK,* and E. M. GARDENHIRE‡

Advanced Monitoring Development Group, Health and Safety Research Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6101 (T. V.-D., G. D. G., K. R. A.); and Department of Chemistry, University of Tennessee, Knoxville, Tennessee 37996-1600 (B. J. T., M. J. S.)

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-μL 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 simpler, more practical, but equally sensitive and selective analytical instrumentation.

The use of fiberoptics-based sensors has recently been reviewed.4–6 For the past few years our laboratories have been involved in the development of various fiberoptics-based monitors.8–11 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 a 600-μm-diameter, multimode, 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
three-step synthesis, following an established procedure. In order to produce anti-BaP antibodies, one couples BaP molecules to a carrier protein, directing the antibodies immobilized on the sensor tip. 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 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 procedure. 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. 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 (NH₄)₂SO₄ precipitation and DEAE-cellulose chromatography.

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-glycidoxypropyltrimethoxysilane (GOPS), with the use of previously described procedures. 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 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.

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 procedure.
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. 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.

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 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.
was performed with the use of (1) 6-M KSCN, and (2) 6-M KSCN containing 10% dimethylsulfoxide (DMSO).

In the second experiment, a two-step elution procedure was conducted with only 5-μL 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-μL 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 (~8 x 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.

ACKNOWLEDGMENTS

This research is jointly sponsored by the National Institutes of Health (Grant No. 5R01 GM 34730-02) and the Office of Health and Environmental Research, U.S. Department of Energy, under Contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.

1. Polycyclic Hydrocarbons and Cancer, H. V. Gelboin and P. O. P. Ts'o, Eds. (Academic Press, New York, 1978).
2. R. S. Yalow and S. A. Berson, J. Clin. Invest. 39, 1157 (1960).
3. W. R. Seitz, Anal. Chem. 56, 16A (1984).
4. J. I. Peterson and G. G. Vurek, Science 224, 123 (1984).
5. T. Vo-Dinh and R. B. Gammage, Amer. Ind. Hyg. J. 42, 112 (1981).
6. B. J. Tromberg, J. F. Eastham, and M. Sepaniak, Appl. Spectrosc. 38, 38 (1984).
7. T. Vo-Dinh, G. D. Griffin, and K. R. Ambrose, Appl. Spectrosc. 40, 696 (1986).
8. M. J. Sepaniak, B. J. Tromberg, and J. F. Estham, Clin. Chem. 29, 1678 (1983).
9. T. Vo-Dinh, G. D. Griffin, K. R. Ambrose, M. Sepaniak, and B. J. Tromberg, "Fiberoptics-Based Immunofluorescence Spectroscopy for Monitoring Exposure to Polynuclear Aromatic Compounds," delivered at the 10th International Symposium on Polycyclic Aromatic Hydrocarbons, Battelle, Columbus, Ohio (1985).
10. B. J. Tromberg, M. J. Sepaniak, T. Vo-Dinh, and G. D. Griffin, "Fiberoptic Chemical Sensors for Competitive Binding Immunoassay," Anal. Chem. (in press).
11. R. M. Sutherland, C. Dahne, J. F. Place, and A. S. Ringrose, Clin. Chem. 30, 1533 (1984).
12. H. J. Creech, J. Am. Chem. Soc. 63, 576 (1941).
13. H. J. Creech and R. N. Jones, J. Am. Chem. Soc. 63, 1681 (1941).
14. G. D. Griffin, K. R. Ambrose, R. N. Thomason, C. M. Murchinson, M. McManis, P. G. R. St. Wrecker, and T. Vo-Dinh, "Production and Characterization of Antibodies to Benzo(a)Pyrene," delivered at the 10th International Symposium on Polycyclic Aromatic Hydrocarbons, Battelle, Columbus, Ohio (1985).