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Direct Electrochemical Immunosensor for Polychlorinated Biphenyls

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A direct electrochemical immunosensor has been developed for the determination of polychlorinated biphenyls (PCBs) in water. The assay is based on the measurement of the current due to the specific binding between PCB and anti-PCB antibody-immobilized conducting polymer matrix. The linear dynamic range of the immunosensor is between 0.3 and 100 ng/mL with a correlation coefficient of 0.997 for Aroclor 1242. The method detection limits for Aroclors 1242, 1248, 1254, and 1016 were 3.3, 1.56, 0.39, and 1.66 ng/mL respectively, and at a signal-to-noise (S/N) ratio of 3. The immunosensor exhibited high selectivity for PCBs in the presence of potential interference such as chlorinated anisoles, benzenes, and phenols. The highest cross-reactivity measured for chlorinated phenolic compounds relative to Aroclor 1248 was less than 3%. Recoveries of spiked Aroclors 1242 and 1254 from industrial effluent water, rolling mill, and seafood plant pretreated water at 0.5 and 50 ng/mL ranged from 103 to 106%. The effect of ionic compounds on detection indicates that no significant change in immunosensor signal was observed within the uncertainty of the assay procedure. The detection method may be applied for continuous monitoring of effluent such as waste streams and groundwater.

Introduction

The persistence of polychlorinated biphenyls (PCBs) and their widespread usage are of great environmental concern (*1*). PCBs were commercially produced under the trade name Aroclors. Aroclors are a mixture of chlorinated biphenyls characterized and numbered by the total percentage of chlorine in the mixture. Several methods have been proposed or developed for the determination of PCBs. These include gas chromatography with electron capture detector, mass-selective detectors, and mass spectrometers (*2*). Although these methods are accurate and reliable, they are often tedious and too expensive for large-scale screening purposes, and they utilize large volumes of expensive and toxic solvents.

Some less expensive but more widely available field screening techniques for PCBs and other priority pollutants are provided through a number of colorimetric kits, enzyme immunoassays (EIAs), and biosensors (*3*). These field technologies are known to be capable of generating higher sample throughputs with a substantial decrease in the time required to perform traditional laboratory analyses. Among these field analytical techniques, chemical sensors and biosensors are especially suitable for rapid, continuous, and in-situ on-site analysis of environmental pollutants.

The development of numerous immunochemical methods for the measurement of antigen−antibody (Ab−Ag) reactions continues to be the subject of several research and development efforts. Notable among these are the enzyme-linked immunosorbent assay (ELISA) (*3, 4*), fluorescence (*5*), chemiluminescence (*6*), electrochemical potential measurements (*7*), and piezoelectric and surface plasmon resonance (*8*). Capacitance changes at electrode−electrolyte interfaces resulting from Ab−Ag recognition have been used to develop electrochemical immunosensors (*9, 10*). Also, the voltammetric (*11*), amperometric (*12*), and potentiometric (*13, 14*) detection of Ab−Ag interactions have been accomplished through the application of redox-modified antigens or antibodies-to-electrode interfaces in the presence of analyte substances.

The first essential step in immunosensor development is the immobilization of an antibody to the surface of a transducer using electrochemical, optical, or piezoelectric methodologies. This step can be carried out by a covalent attachment, physical adsorption, or electrostatic entrapment in a polymer matrix. The polymeric support contains a thin-layer reagent such as antibody or antigen that is very essential to the detection system and is also used to increase the number of biological molecules at the sensor surface.

In many cases, irreversible adsorption or covalent bonding of an antibody (Ab) or antigen (Ag) does not lead to an effective immunosensing system due to the instability of the Ab or Ag molecules, low Ab loading, or the potential loss of antigenic activity. A more favorable approach is the incorporation of Ab or Ag molecules in an electrically conducting polymer layer. Polypyrrole (PPy) is suitable for this purpose because it can be easily prepared on miniaturized components and also because it has a high conductivity and is relatively stable. Many PPy-based systems have been described using enzymes, antibodies, catalytically active dopants, ions, or small organics (*14*).

We have previously reported the use of conducting polymers and the application of pulsed amperometric detection (PAD) with flow injection analysis (FIA) to immobilize antibodies (*15*−*17*). In those works, the detection limits of the resulting sensors were in the sub-ppm levels while the selectivity as tested against other proteins was less than 3%. However, the use of pulsed potential waveforms with FIA has other significant advantages such as the reusability of the surfaces obtained (*16, 17*). This results in a rapid, re-usable and low-cost immunosensor. The initial success of demonstrating this approach for the detection of large antigen biomolecules such as human serum albumin (HSA) and thaumatin proteins has now stimulated the use of this technology for low molecular weight (LMW) environmental compounds. In this paper, we describe the development of a rapid immunosensing method for PCBs using conducting polymer electrodes. The method is based on the measurement of changes in current due to the specific binding of the analyte to an improved antibody-immobilized conducting polymer matrix.

The performance of a direct immunosensor in detecting a quantity relating to mass per surface area requires that the antibody binding constants be equal to (or greater than) the inverse of the analyte concentration in order to create a sufficient signal (*18*). Therefore, an important requirement

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for a direct LMW assay is an antibody that is equal to or exceeds the inverse of the analyte concentration. Since antibody binding constant is directly related to the analyte concentration, a surface with a limited binding constant has only limited theoretical dynamic range. This also requires that the conducting antibody membrane retains sufficient activities as well as electrical conductivity.

In this work, conducting antibody membrane was synthesized by a unique electrochemical assembly of pyrrole in the presence of a polyclonal anti-PCB antibody. The resulting sensors were compared with those prepared through passive protein adsorption and physical entrapment. The optimization conditions involved a very careful consideration of the immunoreagent concentrations, electrodeposition conditions, the pH, and the nature of the buffer. The sensor performance in the identification and quantitation of PCBs as Aroclors is reported in this study. The application of the immunosensor for the measurements of environmental water samples and simulated soil samples was carried out. These sensors should enable an efficient, large-scale screening of pollutants, thereby decreasing costs and providing rapid turnaround time.

Pulsed-Accelerated Immunoassay Techniques (PAIT). The immunoreaction detection technique employed in this work is referred to as pulsed-accelerated immunoassay techniques (PAIT). The sensor can be used for analyte detection in a static or flow injection mode by applying pulsed potentials between the sensor surface (or working electrode) and the reference electrode. The current obtained can be directly related to the rate of electrochemical reactions occurring at the working (or sensing) electrode, which in turn may be related to the concentration of the analyte in solution. It is therefore imperative to identify and control the conditions that define the rate-determining steps of the overall electrolytic process. Since the rate of the heterogeneous electron transfer process occurring directly at an electrode can be controlled by varying the applied pulsed potential, then it is possible to choose a potential such that the current is not limited by heterogeneous electron transfer even if this process is electrochemically irreversible. Under these conditions, the relevant rate-determining steps may be controlled by diffusion (i.e., mass transfer), adsorption, or kinetic processes, respectively. At a certain point in time, the sensor signal represents the sum of the contributions from the conductivity of the bulk solution, ion-exchange redox reaction at the polymer matrix, and the charging and uncharging currents induced as a result of pulsing. This can be represented by

$$i_{\text{total}} = i_{\text{cs}} + i_{\text{ex}} + i_{\text{cd}} + i_{\text{A}} \tag{1}$$

where i_{cs} is the current due to change in conductivity of the solution causing ohmic current flow, i_{ex} is the current due to change in ion-exchange redox reaction (cation-controlled) at the polymer matrix, i_{cd} is the current due to charging and discharging induced by pulsing, and i_{A} is the current due to the binding of the analyte to the immobilized antibody. The change in signal level with respect to time is given by

$$\frac{\mathrm{d}i}{\mathrm{d}t} = \frac{\mathrm{d}i_{\text{cs}}}{\mathrm{d}t} + \frac{\mathrm{d}i_{\text{ex}}}{\mathrm{d}t} + \frac{\mathrm{d}i_{\text{cd}}}{\mathrm{d}t} + \frac{\mathrm{d}i_{\text{A}}}{\mathrm{d}t} \tag{2}$$

Except for a short period of time before and after injection when running buffer is exchanged for sample and vice versa, $\mathrm{d}i_{\text{cs}}/\mathrm{d}t = 0$. Since i_{cs} is constant, the cation-controlled ion-exchange redox reaction is also constant, hence $\mathrm{d}i_{\text{ex}}/\mathrm{d}t = 0$. When the current is sampled at the end of the pulse, the component due to charging is significantly reduced or close to zero, and the level of i_{cd} can be treated as a constant. Under these circumstances, the change in the response level reflects the binding of the analyte, and this can be expressed as

$$\frac{\mathrm{d}i}{\mathrm{d}t} = \frac{\mathrm{d}i_{\text{A}}}{\mathrm{d}t} \tag{3}$$

For a simple one-to-one interaction between the immobilized antibody and the antigen in solution to form a complex AbAg, the net rate of the complex formation is given by

$$\mathrm{d}[\text{AbAg}]/\mathrm{d}t = K_{\text{a}}[\text{Ab}][\text{Ag}] - K_{\text{d}}[\text{AbAg}] \tag{4}$$

where K_{a} is the association rate constant (in $\text{M}^{-1}\,\text{s}^{-1}$), and K_{d} is the dissociation rate constant (in s^{-1}). By substituting $[\text{Ag}]_0 - [\text{AbAg}]$ for [Ag], where $[\text{Ag}]_0$ is the total concentration of reactant Ag, we have

$$\mathrm{d}[\text{AbAg}]/\mathrm{d}t = K_{\text{a}}[\text{Ab}]([\text{Ag}]_0 - [\text{AbAg}]) - K_{\text{d}}[\text{AbAg}] \tag{5}$$

The current response I_{A} will then correspond to the amount of [AbAg] complexes formed, and hence the concentration of the bound analytes. Therefore, the concentration of the AbAg complex is related to the concentration of bound Ag, and the current response corresponds to the amount of AbAg complex formed. The maximum response is proportional to the surface concentration of the Ag. Hence, the rate equation can be rewritten as

$$\frac{\mathrm{d}I_{\text{A}}}{\mathrm{d}t} = k_{\text{a}}C(I_{\text{max}} - I_{\text{min}}) - k_{\text{d}}I \tag{6}$$

where $\mathrm{d}I_{\text{A}}/\mathrm{d}t$ is the rate of formation of surface complex; C is the concentration of analyte in solution, which is constant; and I is the sensor signal. By rearranging the rate equation, it can be shown that the deviation of the binding curve is linearly related to the sensor response. The slope of the binding curve is then used to measure analyte concentration. K_{a} and K_{d} can be calculated from the straight line obtained by plotting $\mathrm{d}i_{\text{A}}/\mathrm{d}t$ versus I. The selectivity of the sensor is greatly dependent on the immobilized molecules and the appropriate choice of applied potential. The incorporation of the manipulative steps such as the use of applied pulsed potential (employed here) opens the door for regeneration of the antibody.

Stoichiometric calculations of the complexes formed can be made if the calibration is performed by injecting different unlabeled molecules in series, and each interaction quantified. Suppose, however, that several complexes can form, then we have

$$\text{Ab} + 2\text{Ag} \rightleftharpoons \text{AbAg}_2 \tag{7}$$

If one complex (say, AbAg_2) predominates, the method of continuous variation can be used to identify the stoichiometry of the predominant complex (*19*−*21*). For the reaction Ab + nAg, i.e.

$$\text{Ab} + n\text{Ag} \rightleftharpoons \text{AbAg}_n \tag{8}$$

it can be shown that $[\text{AbAg}_n]$ reaches a maximum when the initial concentrations have the ratio $[\text{Ag}]_0 = n[\text{Ab}]_0$. To show this, we write

$$K = \frac{[\text{AbAg}_n]}{\{([\text{Ab}]_0 - [\text{AbAg}_n])([\text{Ag}]_0 - n[\text{AbAg}_n])\}} \tag{9}$$

and set the partial derivatives $\partial[\text{AbAg}_n]/\partial[\text{Ab}]_0$ and $\partial[\text{AbAg}_n]/\partial[\text{Ag}]_0$ to zero. The maximum signal is reached at the

composition corresponding to the stoichiometry of the predominant complex, that is when the fraction of Ag equals $n/(n + 1)$. Using PAIT, the process of association and dissociation can therefore be monitored, thus providing a basis for kinetic studies while the regeneration of the sensor surface can be performed by reversing the applied potential, thus enabling reusable surfaces in-situ.

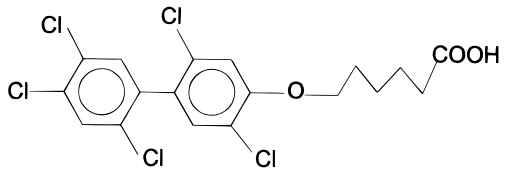
Immunosensor Format and Sensitivity Considerations. The binding of Ab to Ag is based on the law of mass action and with the Ab affinity expressed by the binding constant K (L/mol). So, the performance of a direct immunosensor in detecting a quantity relating to mass per surface area is strongly influenced by the binding constant. Hence, a high binding constant of the antibody toward the analyte is required to achieve a low detection limit in the assay. The degree of surface coverage of an Ab-coated surface at equilibrium conditions (q_{eq}) in relation to the antigen concentration [Ag] and the dissociation constant K_d is given by the Langmuir isotherm as

$$q_{eg} = [\mathrm{Ag}]/\left([\mathrm{Ag}] + \left[\frac{1}{K}\right]\right) \qquad (10)$$

If the concentration of the reactant in solution is lower than the inverse of the binding constant, then the equilibrium coverage will be greatly reduced. Although labeled immunoassays will still work under this condition, the performance of a direct immunosensor will be hindered. Hence the amount of antibody incorporated must exceed the minimum concentration of analyte to be measured.

Experimental Section

Reagents and Standard Solutions. *Antibodies for PCBs.* Anti-PCB antiserum, pool AC-3, were produced under EPA Contract 68-03-3511 and obtained from Dr. J. M. Van Emon's group (*22*), and the procedure employed is summarized: The rabbit polyclonal antibodies produced against a 4-hydroxy analogue of 2,2′,4,5,5′-pentachlorobiphenyl, namely, 6-2,2′,4′,5,5′-pentachloro-4-biphenylol)hexanoic acid. This analogue was covalently linked to keyhole limpet hemocyanin. The coating antigen was prepared from 4-(2,4,5-trichlorophenoxy)butyric acid and bovine serum albumin using a modified procedure described by Langorne et al. (*23*).



(11)

In this modified procedure, 1-ethyl-3-((3-dimethylaminopropyl)carbodiimide was substituted for *N*,*N*′-dicyclohexylcarbodiimide. The antibody was purified using Protein A affinity Column and desalted over an Excellulose desalting column (Pierce). Protein concentration was monitored by absorbance at 280 nm. Antibodies were divided into aliquots and stored in a deep freezer (−18 °C) for later use.

Pyrrole was purchased from Aldrich Chemical Co. (Milwaukee, WI) and was distilled prior to use. It was covered with aluminum foil and stored in the refrigerator to prevent UV degradation. Aroclor standards 1016, 1242, 1248, 1254, 1260, and 1268 were purchased as 1×10^{-4} g/mL solutions in methanol from Ultra Scientific North Kingstown, RI. A pesticide-grade methanol was obtained from Fisher Scientific (Pittsburgh, PA). Sodium salts of nitrates, phosphates, chlorides, and carbonates of analytical grade purity were obtained from Aldrich and were used in preparing the stock solutions. The antibody solution was prepared by dissolving 100−160 μL into a 25-mL standard flask in Nanopure water and was prefiltered through Whatman No. 541 filter paper prior to use. The supporting electrolytes were 0.1 M NaCl and 0.1 M phosphate buffer (pH 7.4). Since PCBs are dangerous toxic compounds, precautions were taken to prevent accidental release, and proper disposal procedures were observed. Fresh solutions were prepared in a pH 7.4 phosphate buffer. Standard reference materials (SRMs) were obtained from Environmental Resource Associates; the commercial soil extraction kit was from EnviroGard. Water samples was obtained from the U.S. EPA sample control center in Virginia.

The following solutions were used for enzyme linked immunosorbent assay (ELISA) experiments. These include anti-PCB anti-serum, pool AC-3, anti-rabbit IgG-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO), and coating antigen (Co-Ag); 4-(2,4,5-trichlorophenoxy)-butyric acids were conjugated to bovine serum albumin (BSA), which was synthesized as described in ref 22. The substrate used was Sigma No. 104 phosphate substrate tablets. The following buffers were used for the preparation of standards and enzyme tracer dilution: carbonate coating buffer with pH 9.6; 1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$, and 0.2 g of NaN_3 dissolved and diluted to 1 L; phosphate buffer saline with Tween 20 pH 7.4; 8.0 g of NaCl; and 0.2 g of KH_2PO_4. The Aroclor primary standards were diluted serially 1:2 into pesticide-grade methanol using 10-mL class A volumetric flasks and 5-mL class A volumetric pipets to obtain ELISA standards.

Instrumentation. The electrochemical equipment consisted of a computer-controlled Cypress Systems Model CS 1090 potentiostat (Kansas City, KS) together with an Epson LX printer for data collection. Galvanostatic polymerization was achieved with the aid of Amel Galvanostat Model 2053 (New York). FIA was performed using Dionex (Sunnyvale, CA) pulsed amperometric detector (PAD-2) together with an analytical pump (APM-1). A thin-layer amperometric cell was controlled by the Dionex PAD detector, and the output current was recorded on a strip chart recorder. A three-electrode voltammetric cell was used for all polymerization. Platinum and silver were used as substrates for the electropolymerization and entrapment of the antibody. The reference electrode was Ag/AgCl (3 M KCl) while the auxiliary electrode was platinum wire at a constant potential of 0.7 V. In all cases, bare substrates were pretreated by the application of +0.2 V for 2 min before polymerization and then followed by −0.5 V for 2 min in 0.1 mol/L sulfuric acid. The electrodes were thoroughly rinsed with the buffer after the immobilization process. All solutions were deoxygenated by bubbling nitrogen through the solution for at least 10 min prior to synthesis of the polymer.

The ELISA apparatus was comprised of Maxisorb I-96 well microplate, automated plate washer (Skatron Model A/S), microplate shaker (Bellco mini-orbital shaker), and microplate reader Model Vmax (Molecular Devices). The instrument control and data analysis software for the plate reader was Molecular Devices Softmax. Pipets used were capable of delivering 1 mL and were adjustable 20−200 μL single-channel pipet, 0−25 μL adjustable positive displacement pipet, 8-channel 50−200 μL adjustable pipet, borosilicate glass tubes (12 × 75 mm or 13 × 100 mm), and acetate plate sealing tape (Dynatech, VA). Mechanical plate shaker and Bellco mini-orbital shaker were from Bellco Biotechnology (Vineland, NJ).

Preparation of Antibody Membrane Electrode. Antibody membrane electrodes were prepared using three main formats as follows (Figure 1):

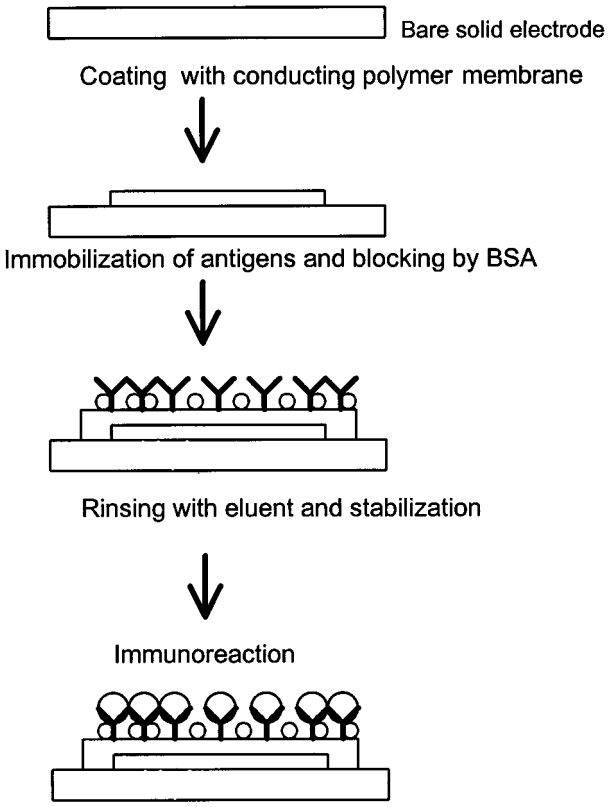


FIGURE 1. Schematic diagrams of the procedures for preparation and measurement. Polymers were prepared as described in the Experimental Section.

Electrochemical microassemly. PCB immunosensors were prepared by constant potential (0.7 V) electrochemical deposition from a 0.2 M pyrrole monomer solution onto platinum or silver substrates. An antibody (54 mg/mL IgG concentration) raised against PCBs served as the counterion in the polymerization process. Blank polymers were prepared with simple inorganic electrolytes containing no proteins. Antibody layers were further adsorbed onto the pyrrole layer by dipping the PPy/$H_2PO_4^-$ electrodes into a solution containing 100 μL of PCB antisera, which was made up in PBS buffer (pH 7.4) for 60 min.

Physical Entrapment. Electrochemical polymerization of PPy/antibody membranes was carried out according to previous reports (*16, 17*). The films were grown using a charge of 160 mC, and the thickness of the antibody layer was controlled by the charge passed during polymerization.

Passive Adsorption. A silver or platinum substrate (0.3 cm^2) was preconditioned as described above and was used as the base electrode. The antibody layers were also adsorbed onto the substrates by dipping the electrodes into a solution containing 100 μL of PCB antisera, which was made up in PBS buffer (pH 7.4) for 60 min. In all these cases, the PPy/antibody electrodes were immersed in a PBS at pH 7.4 for 10 min after preparation to remove the adsorbed proteins and then thoroughly rinsed with PBS at pH 7.4, followed by air-drying. The dried PPy/antibody electrodes were stored covered at 4 °C.

Characterization of Antibody Membrane Electrodes. Antibody membrane electrodes were characterized by using cyclic voltammetry (CV), chronoamperometry (CA), and ELISA protocols. The electrolyte solution used for the CV consisted of 0.1 M phosphate buffer saline (PBS) at pH 7.4, 0.1 M NaCl, and 0.1 M $NaHCO_3$. The ELISA test was conducted to assess the bioactivity of the antibody protein incorporated into the polymers. This was directly performed

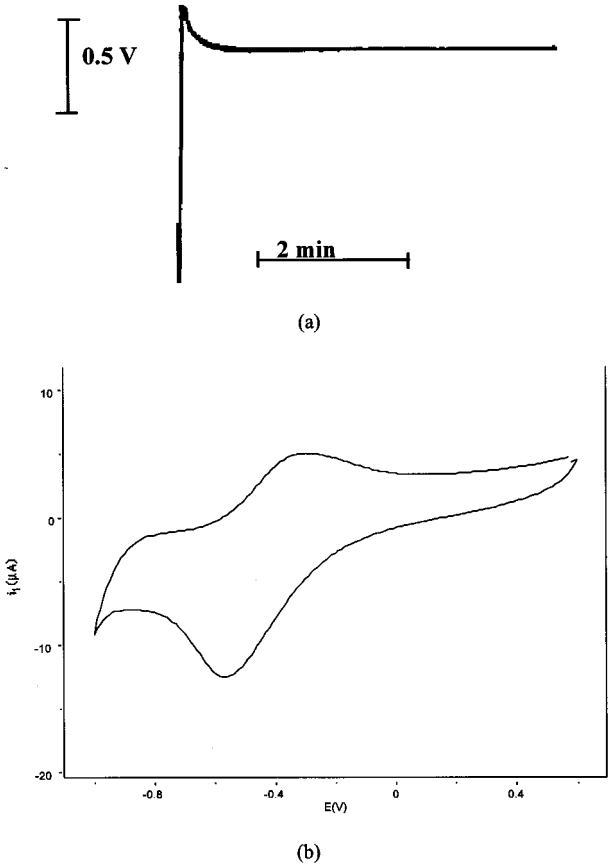


FIGURE 2. (a) Chronopotentiomegram recorded during the electropolymerization of PPy/APCBa. (b) Cyclic voltammogram obtained using PPy/APCBa in phosphate buffer (pH 7.4). Electrode was prepared as in the Experimental Section. Scan rate = 100 mV/s.

on the polymers deposited on platinum strips coated on polyester and prepared as described above. A section of the polymer was removed from the bulk polymer using a hole puncher and was placed in the bottom of the microtiter plate for ELISA analysis. The plates were read at 405 nm at about 10-min intervals.

Surface imaging of the antibody membrane electrodes was carried out using atomic force microscopy (AFM). The AFM imaged polymers were deposited on platinum strips coated with polyester. The films were first plasma-cleaned and then further cleaned by dipping into ethanol to remove surface contaminants before being air-dried. The samples were prepared for imaging by mounting them on scanning electron microscopy-type stubs using double-stick tape. The AFM studies were completed by using a standard Burleigh Instruments, Inc. (Fishers, NY) AFM equipped with an ARIS-3070 70-μm scan module and standard AFM probes. The cross-sectional analysis tools in the True Image software were used to measure the dimensions of the surface features of the antibody electrodes.

Pulsed-Accelerated Immunoreaction. Using the sensor, signal generation was achieved by stationary cell and flow injection analysis (FIA) with pulsed amperometric detection as previously described (*16, 17*). In PAIT, the analytical signal was generated by applying a pulsed waveform between +0.60 and −0.60 V with a pulse frequency of 120 and 480 ms. The oscillating potential reversibly drove the Ab−Ag binding process. The current arising from this process was monitored in real time. After the pulsed accelerated immunoreaction, the electrodes were rinsed with the buffer and reused. An inhibition immunoassay was carried out so as to enhance the changes in current due to the PCB molecules. About 10

TABLE 1. ELISA Results for Polypyrrole−Antibody Electrodes[a]

membrane	total activity (per cm^2 of electrode/mg)
PPy/APCBa	4.235
PPy/APCB$_b$	3.845
APCB layer$_c$	3.116
blank	0.582

[a] PPy, polypyrrole; APCB$_a$, sensors prepared by electrochemical microassembly; APCB$_b$, sensors prepared by direct entrapment techniques; APCB$_c$, sensors prepared by passive adsorption. Total activity refers to the activity units (AU) per cm^2 of polymer. All terms are as described in the Experimental Section.

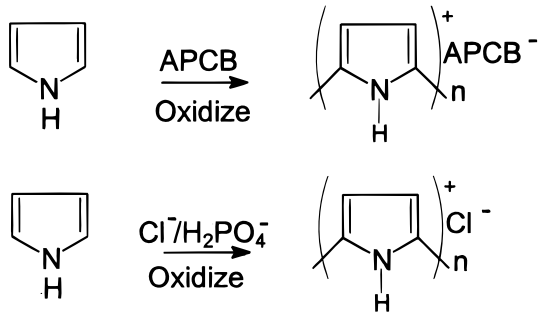
L of the sample or standard solution was mixed with 20 L of protein-labeled PCB standards in PBS buffer, and the solution was allowed to equilibrate for 1 h at 37 °C. Then 25 L of the mixture was injected into the antibody-adsorbed polymer electrodes, which were sufficiently incubated at room temperature. In the stationary format, all current signals were measured at constant temperature and in buffer solutions. The background currents of the bare electrodes were determined as I_0. After the polypyrrole coating and the immobilization of antigen and BSA, the currents were measured as I_1 and I_2, respectively. The current measured after the immunoreaction was I_3. The concentration of the PCBs was determined between I_3 and I_2.

Extraction Studies. Extraction of PCBs from soil and water samples was used to determine the overall significance of the present immunosensor-based analytical method. A simple extraction procedure was employed in order to fully exploit the rapid and simple approach of the immunosensor methodology. Since the solubility of PCBs in water is generally low, a simple liquid−liquid extraction using any water-immiscible solvent is adequate, and thus the extraction procedure employed was derived from known procedures used for field applications (*24*). Basically, the extraction procedure involves placing the sample, drying agent, and extraction solvent in a suitable solvent, followed by mechanical shaking for 20 min at maximum amplitude. Samples were allowed to settle before being filtered using the Envirogard field soil extraction kit. The extracts were used for the analysis using pulsed accelerated immunosensing technique.

Results and Discussion

Polymer Synthesis and Characterization. The electrochemical polymerization provided a simple means of incorporating a range of counterions (C^-) into the polypyrrole according to eq 12. The counterion solutions for polymerization contained the PCB antibody (APCB) or sodium salts of chloride or phosphate ions (Cl^- or $H_2PO_4^-$). The optimization of the counterion concentration was achieved by varying the concentration of the APCB.

The current−time or current−potential readouts during electrochemical deposition were monitored in situ. A constant potential observed during the electropolymerization indicated that a conducting polymer was formed (Figure 2a). Electrochemical characterization of the resulting polymer was carried out using cyclic voltammetry (CV). The CV recorded after polymer growth indicated that an electroactive polymer capable of undergoing reduction and oxidation processes was obtained (Figure 2b).



(12)

where APCB = anti-PCB antibody, Cl^- = chloride and $H_2PO_4^-$ = phosphate counterion. The characterization of the antibody electrode was carried out using ELISA protocols to assess the antibody bioactivity. The polymers used for ELISA experiments were carried out as described in the experimental section. Using the linear portion of the graph of the standard curves of log 1/[APCB] versus absorbance (405 nm), the

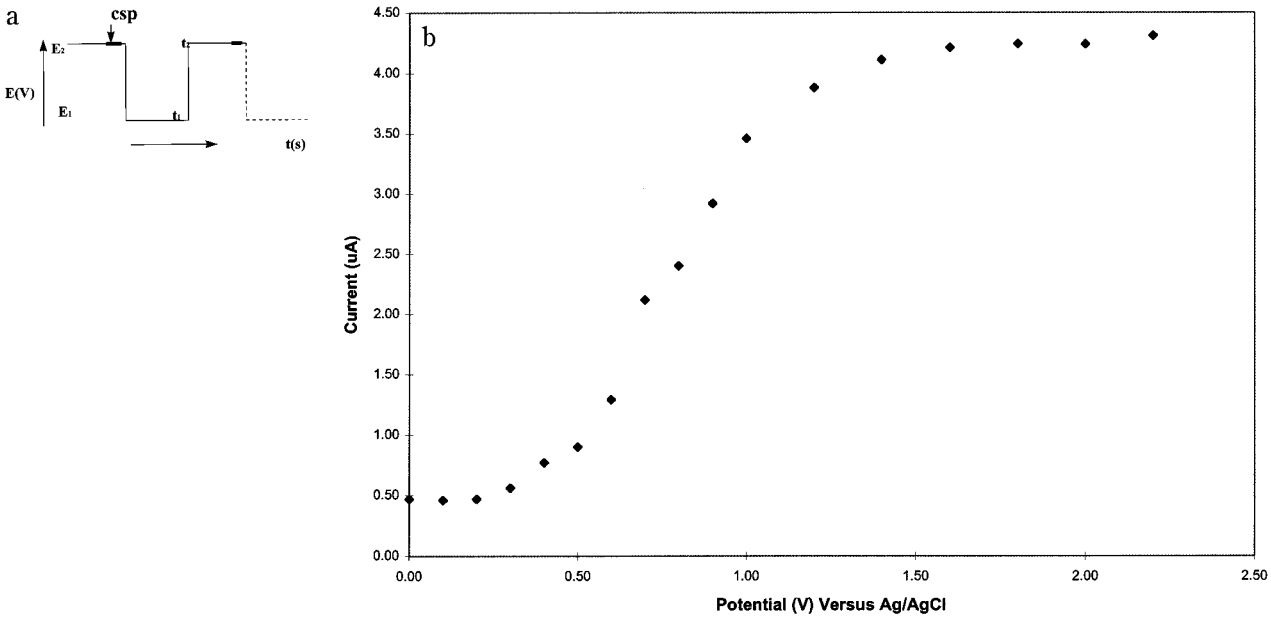


FIGURE 3. (a) Pulsed potential waveform applied, E_1 = initial potential, E_2 = final potential, t_1 = time 1, t_2 = time 2, csp = current sampling point (16.7 ms before the end of pulse. (b) The resulting pulsed potential hydrodynamic voltammogram obtained at PPy/APCBa for A1242. PCB concentration = 50 ng/mL, injection volume = 100 μL, flow rate = 0.5 mL/min, E_1 = 0.6 V, E_2 varies as shown on the *x*-axis, t_1 = 60 ms, t_2 = 480 ms.

absorbance values for the "unknowns" were determined, thus the concentrations of APCB (μg/mL) bound to the coating antigen were calculated. These concentrations obtained were related to the activity by assigning 1 μg of the antibody to be equal to 1 unit of the activity.

The ELISA results showed that the polymers prepared by electrochemical microassembly, e.g., PPy/$APCB_a$, were found to produce the highest activities (see Table 1), and were therefore employed for subsequent experiments. The increased activity at PPy/$APCB_a$ is not surprising because under experimental conditions, the concentration of the adsorbed Ab (and hence the surface coverage) of the PPy/$APCB_a$ electrode is higher. PPy/$APCB_b$ and $APCB_c$ prepared by direct Ab entrapment and passive adsorption methodologies produced activities of 3.845 and 3.116, respectively. The blank values obtained with PPy/$H_2PO_4^-$ were presumably due to nonspecific binding at the electrode. The ELISA experiments thus confirmed that the antibodies were not only incorporated but still retained their bioactivities when immobilized into the conducting polymer matrix.

AFM studies were carried out directly on the surface of the antibody electrodes to assess their electrode surface morphology and thickness. The root mean square (rms) value was used to measure surface roughness, the relationship between the roughness, and the depth. From the cross-section AFM analysis, the depth of the antibody electrode was measured as 1.65 μm, which was consistent with the expected depth of approximately 2 μm. This depth information was directly obtained from the *Z*-value of the AFM image.

Analytical Signal Generation Using PPy/APCB Polymers. The polymer electrodes prepared by electrochemical microassembly, i.e., PPy/$APCB_a$, were used to generate PCB analytical signal using pulsed potential and FIA (Figure 3 a). A pulsed potential hydrodynamic voltammogram (HDV) was obtained by keeping the initial potential (E_1) constant at 0.6 V and varying the final potential (E_2) between −0.1 and −1.5 V (Figure 3b). The current was sampled at the end of E_2. Small signals were recorded as the potential was pulsed negatively between 0.0 and 0.4 V, but these signals increased in amplitudes as the potential was pulsed more negatively. From 0.5 V, a significant rise in signal of about 1.3 V was obtained as the potential-controlled PCB binding at the electrode was recorded. The PPy/$APCB_a$ electrodes were found to exhibit saturation kinetics above 1.3 V. The HDV responses were found to follow similar patterns for all Aroclors investigated, with current magnitude (not shown) decreasing from A1260 > A1254 > A1248 > A1242. This was attributed to changes in the conductivity of the polymer as the percentage of the chlorine levels changed from 60% to 42% (*2*). Since conducting polymers are known to be less conductive at this potential range, this behavior was attributed to an increased polymer porosity, resulting in the accessibility of the active binding sites.

Influence of Applied Potential. Although the affinity of the polymer-bound Ab remains constant, the electrochemical properties of the PPy membrane should largely be influenced by a controlled applied potential. It should be noted that the net charge of the PPy matrix drastically changes depending on the potential (*25, 26*). The dependence of the degree of oxidation−reduction on the applied potential was monitored by reducing the film in a stationary cell for 30 min under the same HDV conditions. The current was found to increase rapidly at an oxidizing potential of 0.6 V and decreased at −1.3 V. During this process, the current through the PPy film dropped from 2.48 to 0.08 μA with an attendant loss in the film conductivity (*25, 26*). As shown in Figure 4, the analyte has a decreased binding preference toward the partially oxidized PPy film. Since the film reduction resulted in a corresponding decrease in the rate of PCB binding, this strongly suggested that the binding interaction was due to positive charges on the oxidized PPy. Therefore, this demonstrates that the antibody recognition of PCB molecules was regulated by the potential of the conductive Ab membrane.

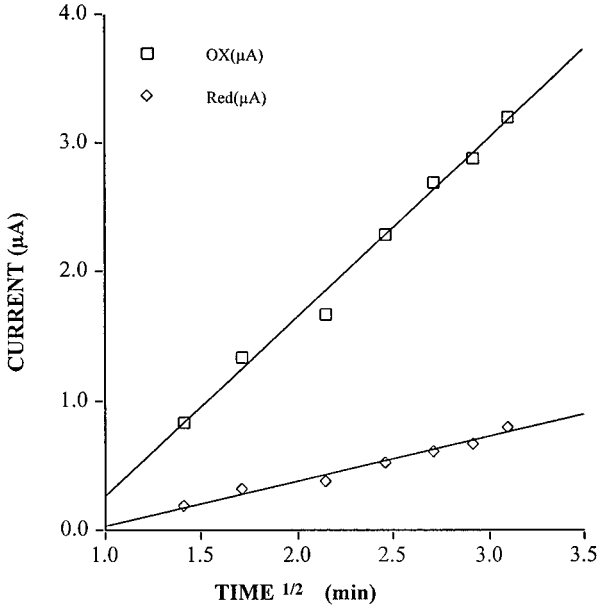


FIGURE 4. Influence of applied potential on binding at PPy/$APCB_a$. Conditions: oxidizing potential = 0.6 V, reducing potential = −1.3 V.

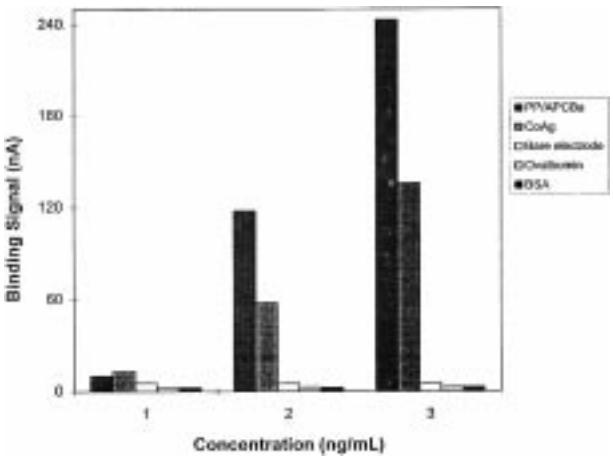


FIGURE 5. Specificity of PPy/$APCB_a$ recognition of PCB at different concentration. E_1 = 0.6 V, E_2 = −0.8 V, flow rate = 0.3/min, t_1 = 60 ms, t_2 = 480 ms.

Specificity of the Current Signal. The specificities measured from the current generated by the PPy/$APCB_a$ for 0.78, 25.0, and 50.0 ng/mL PCB concentrations were determined using electrodes modified with different proteins. The response to different proteins are shown in Figure 5. As expected, blank electrodes (i.e., with no polymer and no immobilized proteins) bound with relatively little PCB, thus resulting in a small current. This nonspecific binding was reduced by nearly half when the bare electrode was coated with 1 mg of ovalbumin made up in PBS prior to use. This suggested that ovalbumin masked some of the sites on the surface of the polymer that bound the PCB. The electrodes coated with anti-PCB coating antigen (Co−Ag) produced significant recognition for the PCB, while the PPy/$APCB_a$ electrode generated significant current above the background due to the accumulation or specific binding of the PCB on the surface.

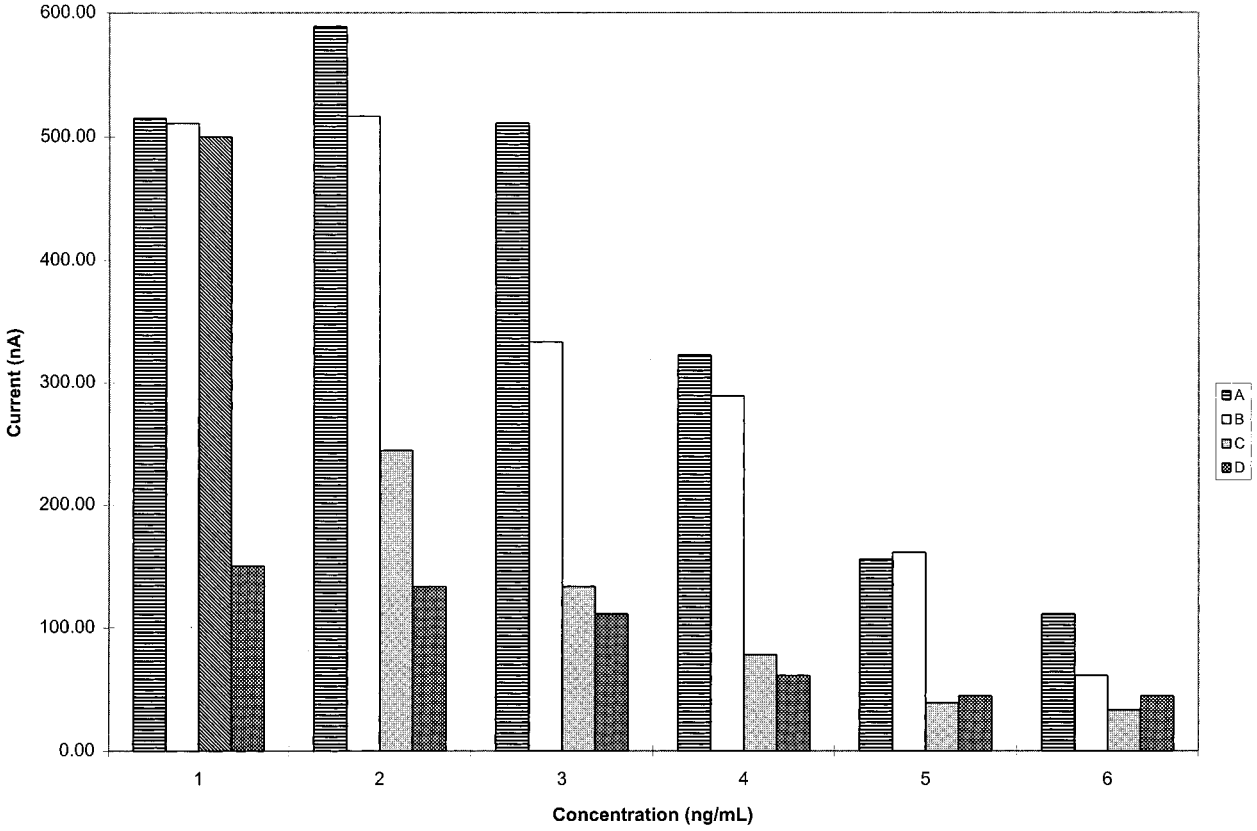


FIGURE 6. Effect of antibody concentration on FIA signals: (a) slope of antibody binding by PCB at 1:250 dilution, (b) 1:500, (c) 1:1000 dilution, (d) 1:2500 dilution. Conditions: 1 = 100 ng/mL, 2 = 50 ng/mL, 3 = 25 ng/mL, 4 = 12.5 ng/mL, 5 = 1.56 ng/mL, 6 = 0.39 ng/mL, E_1 = 0.6 V, E_2 = −0.8 V, t_1 = 60 ms, t_2 = 480 ms, flow rate = 0.5 mL/min.

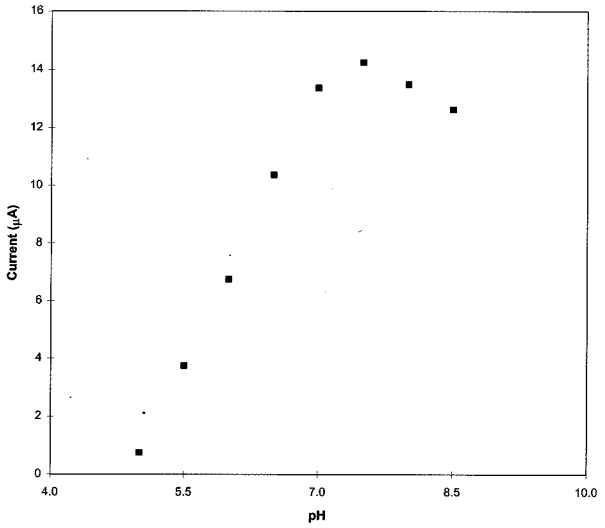


FIGURE 7. Effect of pH on PCB binding at PPy/APCB$_a$ electrode. Antibody dilution = 1:1000. Other conditions as in Figure 6.

Effect of Antibody Concentrations on Current Signal. The current signal generated occurred through the specific binding of the analytes to the immobilized anti-PCB antibodies. Expectedly, when the concentration of the anti-PCB Ab increases, the electrodes containing a distinct number of Ab will accumulate more analytes, and the current signal measured should then increase in a concentration-dependent manner. Thus, the Ab-coated electrodes were exposed to different concentrations of analytes ranging from 0.39 to 100 ng/mL, and the magnitude of the measured signal was found to be concentration dependent. The analyte binding at the anti-PCB polymer electrode was investigated between 1:10 and 1:5000 dilution using 0.39−100 ng/mL of analyte concentrations. The Aroclor standards were loaded into the sample loop of the FIA at 0.3 mL/min flow rate. Currents measured were found to be linear with increasing concentrations of PCBs, but at higher concentration the current signals appeared to saturate. The slope of the resulting binding curves was determined and the results are shown in Figure 6. Antibody concentrations of less than 1:200 dilution provided insufficient sensitivity. Accordingly, a polymer with 1:1000 dilution was selected as the antibody concentration because this gave fairly strong and linear current signals. Antibodies in excess of 1:2500 did not improve the PCB binding in the flowing system. The mean of the standard deviation from all slope values was 0.08 ± 0.0032 nA/s.

TABLE 2. Summary of Results Obtained for Aroclor 1254 at Polypyrrole Antibody Electrode Using Different Buffers

buffer	sensitivity (nA/ng)	correlation coefficient	response time (s)	linear dynamic range (ng/mL)
PBS	452	0.993	34	0.39−100
PBST[a]	460	0.999	38	0.39−100
$NaNO_3$	640	0.916	45	1.00−200

[a] PBST, phosphate buffer saline Tween 20. *n*, an average of 10 injections.

Influence of pH and Nature of Eluent. The efficiency of the binding reaction between the PPy/APCB$_a$ electrode and the PCB analyte was determined by investigating the influence of pH and the nature of the eluent on signal generation. Figure 7 shows the influence of pH on the binding reaction. As the binding slope increases with increase in pH, a peak was formed at around pH 7−8. This confirms the importance of the immobilized APCB molecules in the binding reaction. Therefore, using established pH conditions, the nature of

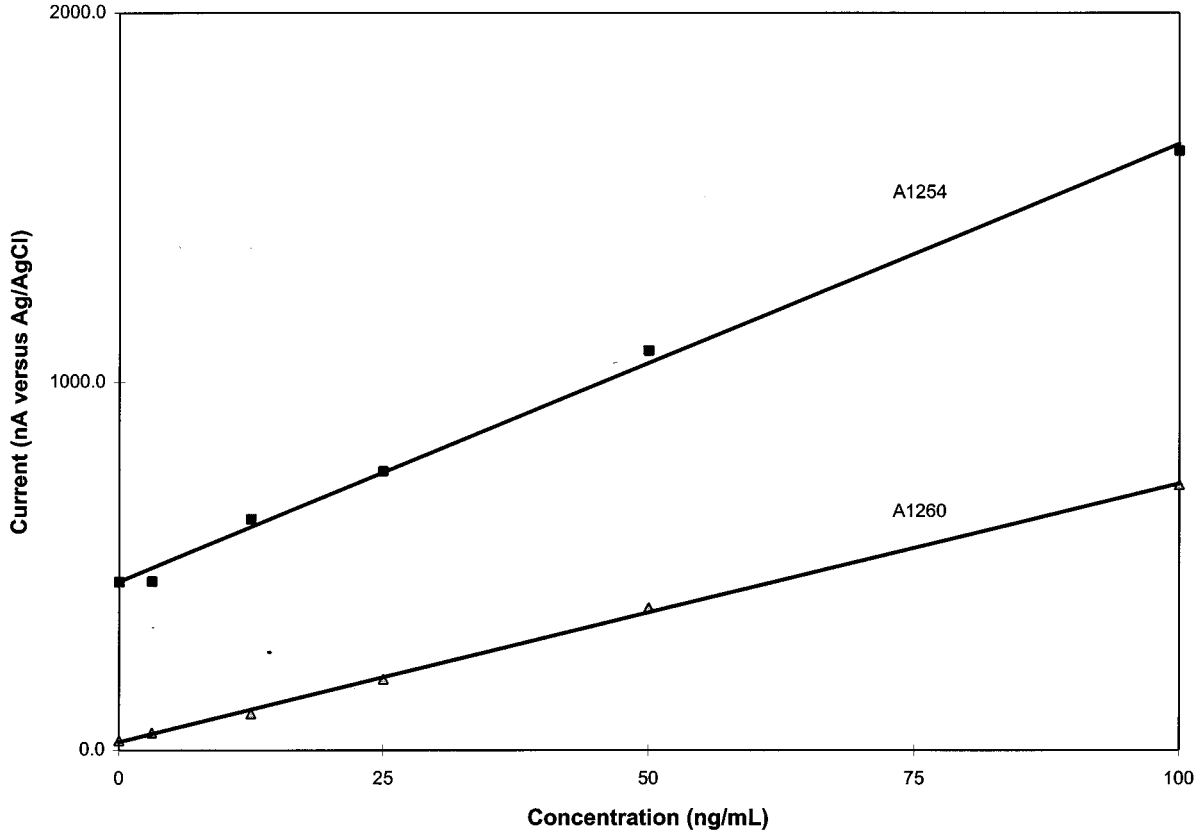


FIGURE 8. Calibration curves obtained for the Aroclors 1260 and 1254 using the PP/APCB$_a$ immunosensor. Conditions: injection volume = 100 μL, flow rate = 0.5 mL/min, E_1 = 0.6 V, E_2 = −0.6 V, t_1 = 60 ms, t_2 = 480 ms.

TABLE 3. Specificity Obtained Using the PCB Immunosensor[a]

compound	MDL[b] (ng/mL)	IC$_{50}$ (ng/mL)	% reactivity
Aroclor 1242	3.33	48.0	52
Aroclor 1260	0.78	54.4	46
Aroclor 1248	1.56	25.0	100
Aroclor 1254	0.39	53.0	47
Aroclor 1016	1.66	68.0	37
Structurally Related Compounds			
anisole, 4-chloro	3.50	>20 000	<0.20
anisole, 2-chloro	3.80	>20 000	<0.20
anisole, 2,3,5,6-tetrachloro	1.60	846	<3.00
biphenyl	200	>20 000	<0.20
benzene, 1,2,3-trichloro	1.12	>20 000	<0.20
benzene, 1,2,3,4-tetrachloro	1.50	>20 000	<0.20
butyric acid, 4-(2,4,5-trichlorophenoxy)	10.0	68 880	0.36
phenol, 2,3-dichloro	145	29 320	<0.10
phenol, 3,5-dichloro	124	1 188	<3.00
phenol, 2,4,5-trichloro	180	869	<3.00
phenol, 2,4,6-trichloro	200	>20 000	<0.20
phenol, 2,3,5,6-tetrachloro	3.8	1 220	2.00
pentachlorophenol	3.5	>20 000	<0.20
DDT	4.9	>20 000	<0.20
DDE	3.2	>50 000	<0.10

[a] Signal generation obtained using (PAIT), E_1 = 0.4 V, E_2 = − 0.8 V, t_1 = 120 ms, t_2 = 480 ms, injection volume = 50 μL, flow rate = 0.5 mL/min, n = average of 10 injections. Injection volume = 50 L, flow rate = 0.5 mL/min. [b] MDL = method detection limit, which is 3 times signal-to-noise (S/N) ratio.

TABLE 4. Recoveries of PCBs from Environmental Samples[a]

matrix	spike level (ng/mL)	measured level (ng/mL)	recoveries[b] (%)
industrial effluent water	50.00	53.00	106 ± 6
	0.50	0.52	104 ± 2
rolling mill pretreated water	50.00	52.00	104 ± 4
	0.50	0.53	106 ± 2
seafood pretreated water	50.00	52.00	104 ± 6
	0.50	0.50	100 ± 6
soil SRMs (A1242)	25.00	25.33	101 ± 2
	8.00	8.02	102 ± 2
	0.50[c]	0.46	92 ± 6

[a] Measured value for spiked sample was calculated as the sum of the known spike value plus the measured value of the unspiked samples. The measured values for the unspiked samples were obtained by PAIT. Water samples were filtered through a 0.45-μm filter, adding the eluent buffer chemicals and then carrying out the assay procedure on each sample. [b] Mean ± standard deviation, n = 6. [c] Expected value.

the buffer employed for the analysis was also investigated. These buffers were phosphate buffer (pH 7.4), phosphate buffer saline (pH 7.4), and sodium nitrate eluent (pH 7.4). Using these buffer systems, well-defined peaks were obtained for the Aroclors in all the eluents. There were however significant differences in the signal magnitude, peak shapes, and detector response times as the eluent composition were changed (Table 2). Although the sensitivity was less as compared to other eluents considered using the PBS buffer, this buffer was chosen for subsequent FIA experiments due to a reduced response time.

Calibration. Linear calibration curves of the peak heights (nA) versus concentrations (ng/mL) were obtained for all Aroclors in all the eluents (Figure 8). The electrochemically assembled PPy/APCBa polymer was used in detecting the Aroclors, and quantitation was made by measuring the peak height of the current signal. All operations were carried out at room temperature. In these experiments, analytical signal was generated by applying a pulsed potential waveform between +0.60 and −0.80 V with pulse frequency of 120 and 480 ms. The oscillating potential reversibly drove the Ab−

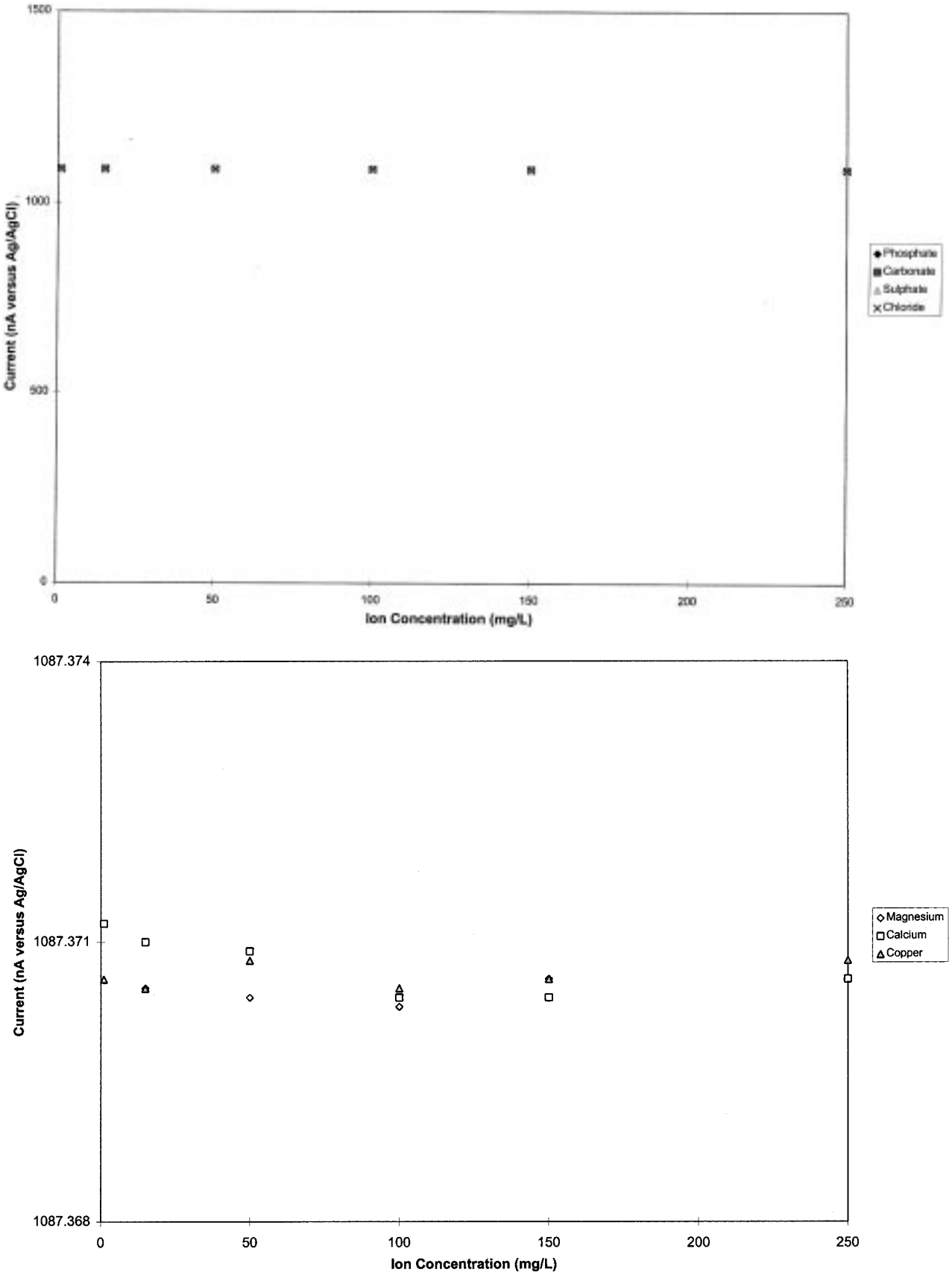


FIGURE 9. Effect of ions on antibody−PCB binding: Antibody dilution 1:1000, 50 ng/mL Aroclor 1254, injection volume 100 μL, flow rate 0.5 mL/min, $E_1 = 0.6$ V, $E_2 = -0.8$ V, $t_1 = 60$ ms, $t_2 = 480$ ms.

Ag binding process. The current arising from this process was monitored in real time. Table 3 is a summary of the calibration data obtained using the immunosensing electrode for the detection of all the Aroclors. Linearity obtained for the Aroclors were in the range of 0.3−100 μg/L with correlation coefficients (r^2) of 0.997 or better. The linear

dynamic range of the immunosensor was 0.3−100 ng/mL with r^2 of 0.998 for Aroclor 1242. The method detection limits for Aroclors 1242, 1248, 1254, and 1016 were 3.3, 1.56, 0.39, and 1.66 ng/mL, respectively. Polymer layers were found to be stable throughout the experimental period because no appreciable reduction in the signals were recorded.

Cross-Reactivity Studies. The degree of binding of the PCBs to some structurally related compounds were examined in order to test for potential cross-reactivity. Since the hapten is linked to the long aliphatic spacer arm via a phenyl−ether linkage, it is possible that chlorophenol ethers might be potentially more reactive than hydroxyl analogues; hence, different substituted phenols, benzenes, and anisoles were examined. These compounds were carried through the standard assay procedure using a series of standard solutions enabling the calculation of IC_{50} values (50% B/B_0). This was calculated as the signal observed for the PCB analyte divided by the signal observed from zero standard. The percentage cross-reactivity is the IC_{50} of the PCB analyte divided by the IC_{50} of each potential cross-reactant (× 100). This value is a direct indicator of the general concentration level at which the system is capable of functioning. The result obtained shown in Table 3 indicated that the highest cross-reactivity measured for the chlorinated phenolic compounds relative to Aroclor 1248 was less than 3%. It appears that the sensor might be highly influenced by nonspecific effects due to the similarities in the reactivity values recorded for the PCBs. A 2.9% cross-reactivity was observed for 2,4,5-trichlorophenol, which is not surprising considering that the 2,4,5-trichloro substitution pattern was present on the immunizing hapten. Since the immunosensor exhibited high selectivity for PCBs, this should enable the sensor to be used in the presence of potential interference such as chlorinated anisoles, benzenes and phenols.

Matrix Interference and Influence of Ionic Compounds. Since most of the assays involving electrochemical immunosensor are to be performed using real-life samples with varying degree of compositions, the extent of interference from other compounds present was determined. In real-life situations, materials may be present in the samples that can partially or completely inhibit assay performance. The presence of some matrix effects was investigated by the recovery (or known addition) technique, where a known quantity of a pure Aroclor standard was added to water samples and the actual change observed was compared with expected values from the knowledge of the amount added. The results obtained for the recoveries of spiked water samples and/or soil standard reference materials using anti-PCB immobilized conducting polymer electrodes are summarized in Tables 4. The measurements of Aroclor 1242 soil standard reference materials (SRMs) were carried out with a recovery of 102% and a relative standard deviation of less than 10%. The recoveries of spiked Aroclors 1242 and 1254 from industrial effluent water, rolling mill, and seafood plant pretreated water at 0.5 and 50 ng/mL ranged from 103 to 106.

$Na_3PO_4 \cdot 12H_2O$, $Na_2HPO_4 \cdot 2H_2O$, NaCl, $NaHCO_3$, $Ca(NO_3)_2$ and $CaSO_4$, NH_4N0_3, and $NaSO_4$ were added to 50 ng/mL of the Aroclors and were injected to test for ionic interference. Up to the concentration tested, no significant change in signal was observed within the uncertainty of the assay procedure as shown in Figure 9. Consequently, the detection method may be used for continuous monitoring of effluents such as waste streams and groundwater so as to measure compliance with regulations.

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