Electrochemical Immunosensors for the Simultaneous Detection of Two Tumor Markers

Michael S. Wilson*

EIC Laboratories, Inc., 111 Downey Street, Norwood, Massachusetts 02062

The microfabrication of electrochemical immunosensors for the simultaneous detection of two protein analytes is described. The sensors consisted of two iridium oxide electrodes (1-mm diameter) patterned on a glass substrate. Capture antibodies were immobilized on the porous iridium oxide electrodes by covalent attachment using (3-aminopropyl)triethoxysilane and glutaraldehyde. The spatial separation of the electrodes (2.5 mm) enabled simultaneous electrochemical immunoassays to be conducted without cross-talk between the electrodes. Proteins were measured using electrochemical ELISA, and detection was achieved by electrochemically oxidizing alkaline phosphatase-generated hydroquinone. Sensors for the simultaneous detection of goat IgG and mouse IgG, and for the tumor markers CEA and AFP, were developed. The sensors had detection limits of 1, 2, 1.2, and 1 ng/mL for goat IgG, mouse IgG, CEA, and AFP, respectively.

Simultaneous multianalyte immunoassays (SMIAs) that can quantitatively measure the concentrations of multiple proteins in a single assay are important new analytical methods. They use less sample, increase test throughput, reduce the cost per test, and improve test efficiency compared to single-analyte assays. Although high-density protein microarrays capable of screening large numbers (100s–1000s) of proteins simultaneously are being developed, quantitative data in these arrays are usually limited or difficult to obtain. This is due to the significant technical challenges posed by the large number of antibody reagents required, the need for characterization of antibody reagents individually and in the complete system, their differing shelf lives, stability and binding affinities, and differing analyte concentration ranges. Because of these issues, arrays for performing accurate quantitative multianalyte analysis are likely to be of much lower density than typical protein microarrays.

Electrochemical immunosensors have gained considerable interest as bioanalytical devices in recent years. They are attractive tools because they are robust, are economical to mass produce, and can achieve excellent detection limits with small analyte volumes. Unlike spectroscopic-based techniques, electrochemical methods are not affected by sample turbidity, quenching, or interference from absorbing and fluorescing compounds commonly found in biological samples. Furthermore, the required instrumentation is relatively simple and can be miniaturized easily to circuit board level with very low power requirements, facilitating the development of disposable devices and methodologies for ultrasmall sample amounts. While the use of electrochemical methods for performing parallel single-analyte immunoassays has been well-established, there has been little application of such devices to SMIA. Electrochemical methods for SMIA that use multiple enzyme labels have been reported, but these methods often involve a compromise in assay conditions since different enzymes may have different requirements for optimum enzyme activity. Furthermore, the use of multiple labels increases the complexity and reduces the convenience of these methods.

A more attractive strategy for electrochemical SMIA is one in which a single enzyme label is used to detect all analytes. This method relies on the spatial resolution between different antibody and electrode locations. In this design, cross-talk can potentially occur due to electroactive product generated at one electrode, diffusing to a neighboring electrode, and producing an interfering amperometric response. Thus, for the simultaneous measurement of two or more analytes, measurements must be recorded from each sensing electrode before cross-talk from neighboring electrodes occurs. An elegant single-label approach to electrochemical SMIA has been described by Meyerhoff et al. This system used nylon membranes containing two gold electrodes, with enzyme-labeled antibody present on one side of the membrane and enzyme substrate present on the other side. The diffusion of substrate through the membrane, and selective measurement of product generated only by electrode-bound enzyme, produced a separation-free immunoassay system. However, the authors noted that the approach would suffer from high background signals when multiple antibodies were used, thus limiting its application in multianalyte detection. Ding et al. have also demonstrated the feasibility of SMIA using a nonimmunoassay format in which different concentrations of enzyme-labeled antibodies were immobilized near electrodes on a polystyrene substrate. Addition of enzyme substrate to the system enabled measurement of the antibody concentrations by recording the current at each adjacent electrode. In work published following the completion of the research described herein, Kojima et al. reported the qualitative detection of two tumor markers using an electrode array. Here,
antibodies were trapped in a siloxane film polymerized on the electrode surface, and detection was achieved using glucose oxidase-labeled antibodies and cyclic voltammetry.

We have recently reported the preparation of electrochemical immunosensors based on antibodies immobilized in an electronically conducting iridium oxide (IrOx) matrix. IrOx has several favorable features for use as a matrix in immunosensor fabrication, providing a porous three-dimensional hydrus environment for immobilized proteins, with a high degree of transport for water, protons, and other ions. In addition, the conducting nature of IrOx enables the oxide to act as both the transducer and immobilization matrix in these devices. We are interested in developing electrochemical SMIA sensors based on arrays of IrOx immunosensing electrodes. It is anticipated that use of the three-dimensional IrOx matrix will be advantageous in SMIA development because diffusion of electroactive enzyme-generated product is hindered in the matrix. This will likely enable the highly efficient capture of enzyme-generated product by the electrode, leading to less cross-talk between sensors, and may allow for the development of smaller devices containing sensors that are closer together than could be achieved using planar two-dimensional electrodes.

Here we describe the design, fabrication, and application of novel SMIA sensors using IrOx matrices that offer multiple benefits in the electrochemical detection of target analytes. We demonstrate the quantitative detection of two model IgG analytes, goat IgG and mouse IgG, as well as two important tumor markers, carcinoembryonic antigen (CEA) and α-fetoprotein (AFP). Our work extends previous concepts in electrochemical-based immunosensors and provides an important foundation for the future development of multianalyte detectors. We anticipate that chip-based sensors, as described herein, will be suitable for the mass production of economical, miniaturized multianalyte assay devices.

EXPERIMENTAL SECTION

Apparatus. Electrochemical measurements were carried out using an AFCBPI two-channel potentiostat (Pine Instrument Co., Grove City, PA). Recording was achieved digitally using one BT 2000 recorder (Bascom-Turner Instruments, Norwood, MA) for each potentiostat channel. Electrochemical experiments were conducted using a three-electrode format with a 1-cm² Pt flag counter electrode and a single-junction Ag/AgCl/3M NaCl reference electrode (MP2063, Bioanalytical Systems, Inc., West Lafayette, IN).

Reagents. Reagents were purchased from the following companies: polyclonal donkey anti-goat IgG, donkey anti-mouse IgG, alkaline phosphate-labeled anti-goat IgG (AP-anti-goat IgG), AP-anti-mouse IgG, goat IgG, mouse IgG, and IgG-free bovine serum albumin (BSA) from Jackson Immunoresearch (West Grove, PA); polyclonal goat anti-human CEA from BiosPacific, Inc. (Emeryville, CA); polyclonal goat anti-human AFP from Fitzgerald Industries International, Inc. (Concord, MA); CEA and AFP from Cortex Biochem (San Leandro, CA); EZ-Link sulfo-NHS-LC-LC- Biotin kit from Pierce (Rockford, IL). Hydroquinone diphosphate (HQDP) was prepared following literature procedures. All other chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI) and used as received.

Buffers. Water was purified using a Millipore MilliQ A10 system (Bedford, MA). Blocking buffer consisted of 100 mM Tris-HCl, pH 7.2, 1% BSA. Reaction buffer consisted of 100 mM Tris-HCl, 10 mM MgCl₂, pH 9.0. PBS consisted of 100 mM phosphate-buffered saline, pH 7.2.

Sensor Fabrication. Sensors for performing two-analyte immunoassays were fabricated as follows. The substrate (Corning 1737 glass, 20 mm × 30 mm × 1 mm) was spin-coated with positive photoresist (Shipley 1818, 5000 rpm, 30 s) and soft-baked at 90 °C for 45 min. Next, the photoresist was patterned by photolithography (UV exposure: 400 nm, 56 mW/cm², 5 s). Development: Shipley CD-30, 1 min). Iridium was deposited on the substrate by dc magnetron sputtering using separately controlled titanium and Ir sources. The Ir (~100 nm) was deposited over a graded Ti–Ir underlayer (~25 nm) for adhesion. A liftoff procedure (ultrasonication in acetone) was then used to strip the photoresist, leaving behind the patterned Ir electrodes (W1 and W2, 1 mm in diameter, 2.5-mm edge-to-edge separation) and electrical connections. A second photoresist procedure was performed, and an insulating silicon carbide layer was deposited over the array using plasma-enhanced chemical vapor deposition. A final liftoff procedure provided the pattern of exposed electrode areas and contacts. Next, the electrodes were immersed in PBS (4 mL), and activated with 0.1-Hz square wave potential pulses with limits of ~600 and +800 mV versus Ag/AgCl (typically 500 pulses). The growth of IrOx was monitored by calculating the charge storage capacity of the oxide using cyclic voltammetry (recorded between ~600 and +800 mV, 50 mV/s scan rate). The electrodes were activated until 20 mC/cm² IrOx had grown (~100 nm thickness). The electrodes were washed with water, followed by ethanol, and dried using a stream of nitrogen. Following the immobilization of antibodies on the electrodes (described below), an acrylic well was attached to the substrate to provide a well for loading solution.

Silane-Mediated Antibody Immobilization. Silanization of the IrOx electrodes was achieved by immersing the electrodes in (3-aminopropyl)triethoxysilane (APTES, 30%) in anhydrous hexane (200 mL) and refluxing under nitrogen for 1 h (Scheme 1). The sensors were then washed with hexane to remove excess silane and dried using a stream of nitrogen. This reaction introduced amine groups onto the surface of the IrOx. Next, the sensors were incubated in a solution of 2.5% glutaraldehyde in degassed phosphate buffer (0.1 M, pH 7.4) under nitrogen for 60 min, rinsed with water, and dried with nitrogen. This reaction introduced aldehyde groups onto the surface of the oxide through reaction of glutaraldehyde with surface-bound amine groups. Antibody solution (0.5 μL, 0.7 mg/mL, 50% glycerol) was then spotted directly onto the desired electrode W1 or W2 using a micropipet and incubated for 60 min at room temperature. This enabled reaction between amine groups on the antibody and surface-bound glutaraldehyde groups. Finally, the sensor was washed with blocking buffer and then incubated in blocking buffer for 60 min. Recently, we have improved the sensor fabrication process by depositing a layer of hydrophobic fluorocarbon polymer (CYTOP, Bellex International, Wilmington, DE) around the electrode sites prior to antibody immobilization. This enables the antibody droplets to be more easily contained within the electrode sites. Reaction between amine and aldehyde usually requires a
**Scheme 1. Attachment of Antibodies to IrOx**

(A) Introduction of amine groups on the oxide surface by reaction with APTES; (B) introduction of aldehyde groups on the oxide surface by reaction with glutaraldehyde; (C) reaction between amine groups on the antibody and oxide-bound glutaraldehyde.

![Diagram showing Scheme 1](Image)

(A) Introduction of amine groups on the oxide surface by reaction with APTES, (B) introduction of aldehyde groups on the oxide surface by reaction with glutaraldehyde, (C) reaction between amine groups on the antibody and oxide-bound glutaraldehyde.

**Preparation of Biotin-Labeled Antibodies.** Anti-CEA and anti-AFP antibodies were labeled with biotin using the EZ-Link sulfo-NHS-LC-LC-Biotin kit following the manufacturer’s instructions (Instruction book 0237dh4).

**CEA and AFP ELISA.** Sensors containing anti-CEA immobilized on electrode W1 and anti-AFP immobilized on electrode W2 were incubated with samples containing either CEA, AFP, or mixtures of both (0–300 ng/mL in blocking buffer, 200 μL, 60 min) with gentle shaking. Next, the sensors were washed with blocking buffer and incubated with a mixture of biotin—anti-CEA and biotin—anti-AFP (1 μg/mL each component in blocking buffer, 200 μL, 60 min, gentle shaking). The sensors were then washed with blocking buffer, incubated with streptavidin-labeled AP (0.5 μg/mL in blocking buffer, 200 μL, 60 min), and washed again with blocking buffer. Amperometric measurements were made in reaction buffer as described above for the IgG assays.

**RESULTS AND DISCUSSION**

**Simultaneous Multianalyte Immunoassay Concept.** The multianalyte immunoassay uses arrays of immunosensing electrodes, each capable of performing an independent electrochemical immunoassay for a specific analyte. Each electrode contains immobilized antibodies and uses an AP-based enzyme-linked immunosorbent assay (ELISA) to measure analyte concentration (Figure 1). Amperometric responses are produced at the electrodes due to the electrochemical oxidation of HQ, and the size of the response enables the quantification of analytes. HQ is generated at the electrodes by the hydrolysis of HQDP by electrode-bound AP. HQDP is a novel AP substrate developed at EIC that does not produce the electrode passivation often associated with other commonly used electrochemical AP substrates. HQ is only generated at electrodes that contain bound target analyte and, therefore, only at electrodes that contain bound target analyte. The assays rely on the spatial resolution between sensing electrodes to enable independent electrochemical immunoassays to be performed without cross-talk between sensors.

Einstein equation, assays to be performed without cross-talk occurring. Using the sufficient distance between sensors for independent immuno-
distance of 2.5 mm (Figure 2). An electrode separation of 2.5 mm
can occur due to HQ generated at one electrode diffusing to an
adjacent electrode and producing an interfering amperometric
response.

The sensors used for performing SMIA for two analytes contain
two IrOx immunosensing electrodes, W1 and W2, separated by a
distance of 2.5 mm (Figure 2). An electrode separation of 2.5 mm
was chosen since it was anticipated that this would provide
sufficient distance between sensors for independent immuno-
assays to be performed without cross-talk occurring. Using the
Einstein equation, \( d = \sqrt{(2D t)} \) (where \( d \) is average distance traveled (cm); \( D \), diffusion coefficient (approximately \( 1 \times 10^{-9} \) cm² s⁻¹ for HQ) and \( t \), time (s)), HQ generated at working electrode W1 or W2
will diffuse on average a distance of 1.5 mm in 20 min. This will
allow ample time for steady-state amperometric responses of the
electrodes to be reached (typically 2–5 min). In addition, since
HQ is only generated in proximity to the electrode surface, it is
likely that most of the HQ will be electrochemically oxidized as
soon as it is formed and will not be available for diffusion away
from the electrode.

**Immobilization of Antibodies on IrOx Electrodes.** We have
previously reported the fabrication of IrOx-based immunosensors
using an in situ antibody immobilization method. In this method,
IrOx is grown by electrochemically activating Ir electrodes in PBS
solution containing antibodies. Antibodies present in the activation
solution become entrapped in the oxide matrix and are available
for antigen binding. This method, however, requires a relatively
high concentration of antibody in the immobilization step for
optimal antibody loading (400 µg/mL) and requires a significant
volume of antibody solution (>100 µL/electrode) to physically
complete the electrochemical cell. Furthermore, if each electrode
is to contain a different antibody, then only one array electrode
at a time can be functionalized. This method of antibody immo-
bilization would, therefore, be expensive and impractical for the
preparation of sensors containing multiple electrodes (>2), with
each electrode containing a different antibody. However, the
reaction of various metal oxides (e.g., titanium dioxide) with
silanes is a well-established method for introducing reactive
functional groups onto oxide surfaces. These functional groups
can then be used to covalently attach proteins. Although the
attachment of antibodies to electrochemically grown IrOx via
silane derivatization has not been reported previously, the use of
such methods to covalently attach glucose oxidase to thermally
produced IrOx using APTES and glutaraldehyde has been
described. Using a similar approach, we developed a more
practical and economical method to immobilize antibodies on the
IrOx electrodes in which antibody solution (0.5 µL) is deposited
directly onto the silanized electrode using a micropipet (Scheme
1). Electrochemically grown IrOx contains a high concentration
of large pores, 50–70 nm in diameter, and it is likely that
antibody is immobilized on the oxide surface throughout these
 pores during the silane immobilization procedure. The pore size
is large enough to allow antigen and enzyme-labeled antibody
(typically 10–15 nm in size) to diffuse in and interact with antibody
immobilized in the pores. This method, therefore, retains the
benefits afforded by the IrOx matrix (a hydrous, porous, three-
dimensional matrix for antibody immobilization), while enabling
the preparation of immunosensors using significantly less antibody
solution compared to the in situ method. Furthermore, the method
can be used easily to simultaneously immobilize different antibod-
ies onto arrays of electrodes. The cyclic voltammogram of the
oxide remains unchanged following reaction with the silane,
indicating that the electrochemical properties of the electrode are
unaffected by silanization.

To evaluate the efficiency of the covalent attachment method,
immunosensors were prepared by immobilizing anti-goat IgG on
the IrOx electrodes using covalent attachment, simple adsorption
(by adding antibody (0.5 µL, 0.7 mg/mL) to untreated IrOx
electrodes), and in situ immobilization. The immunosensors were
used in an ELISA for goat IgG (2.2 µg/mL, 500 µL, Figure 1),
and their amperometric responses were compared. The sensors
prepared by using silane-treated IrOx, nontreated IrOx, and in
situ immobilization produced amperometric responses of 45
(±3.5), 13 (±1.0), and 43 (±3.0) µA/cm², respectively. These
results show that significantly more antibody was immobilized
on silane-treated IrOx compared to untreated IrOx, indicating a
more robust attachment of antibodies and suggesting that covalent
attachment of antibody to the oxide had occurred. The silane-
treated electrode produced amperometric responses comparable
to those obtained with electrodes prepared using the in situ
antibody immobilization method.

**Evaluation of Cross-Talk between Sensors.** To validate the
design of the two-analyte sensor, experiments were performed to
determine whether there was cross-talk between electrodes W1
and W2. Sensors were prepared in which anti-goat IgG was
immobilized on electrode W1, while electrode W2 was treated
with blocking buffer only. The sensors were used in a goat IgG
ELISA (2.2 µg/mL, 500 µL), and the current responses of W1 and W2
were recorded simultaneously. As desired, an immediate current
was observed at W1 (46 µA/cm²), which was the electrode
containing immobilized anti-goat IgG (Figure 3). Conversely, W2,
the electrode containing IrOx only (blocked with BSA), produced
no current response for 40 min following the addition of HQDP.

---

These experiments demonstrated that there was no cross-talk between W1 and W2 and that independent measurements could easily be taken at adjacent electrodes. Approximately 5 min was required for steady-state amperometric responses to be achieved, indicating that the electrodes could be located significantly closer together than 2.5 mm without cross-talk interference.

**Simultaneous Immunoassays for Goat IgG and Mouse IgG.** Sensors for the simultaneous detection and quantification of goat and mouse IgG were prepared by immobilizing anti-goat IgG on electrode W1 and anti-mouse IgG on electrode W2. To verify that the individual assay systems were performing correctly, and to establish that there was no cross-reactivity between antibodies and nontarget IgG, the sensors were used in ELISAs with samples containing varying concentrations of either goat IgG or mouse IgG only. Following incubation with IgG, the sensors were then incubated with solutions of either AP--anti-goat IgG or AP--anti-mouse IgG.

As desired, in assays using goat IgG samples, only electrode W1 produced an amperometric response, and the response increased as the concentration of goat IgG increased (Figure 4A). Electrode W2 produced no current response above background, even as the concentration of goat IgG increased. Similarly, in the assays using samples containing mouse IgG, only electrode W2 produced an amperometric response, and this response increased as the concentration of mouse IgG increased (Figure 4B). In control ELISAs (0 ng/mL IgG), small background responses (0.3–0.4 μA/cm²) due to nonspecific binding of AP--anti IgG were observed on both electrodes. These experiments confirmed that the assay systems were performing correctly and that there was no cross-reactivity between antibodies and nontarget IgG.

To demonstrate the simultaneous multianalyte IgG immunoassay, the sensors were used in ELISAs with samples containing mixtures of both goat IgG and mouse IgG (0–100 ng/mL, 500 μL) and both AP--anti-goat IgG and AP--anti-mouse IgG. In these assays, both W1 and W2 produced amperometric responses that increased as a function of goat IgG or mouse IgG concentration, respectively (Figure 4C).

The dose–response curves of the two-analyte sensor were typical of those exhibited by most immunoassays. Linear regions of pseudo-first-order kinetics (where antibody was in large excess to antigen) were observed between 10 and 55 (goat IgG) and 5–50 ng/mL (mouse IgG). Above these concentrations, nonlinearity due to the onset of antibody saturation was observed. There was good correlation between the performance of the goat IgG sensor in the single- and dual-analyte assays (5–10% difference in responses). However, the mouse IgG sensor gave responses that were 50–60% larger than those obtained in the single-analyte assay. It is unlikely that this behavior was due to binding of goat antibodies to the mouse IgG sensor, since the results of the single-analyte assays (Figure 4A and B) determined that there was no cross-reactivity. Larger amperometric responses due to the formation of a complex between AP--anti-goat IgG and AP--anti-mouse IgG would also be unlikely since all antibodies were raised in the same host (donkey). Furthermore, formation of such a complex would be expected to produce similar increases in response for the goat IgG sensor, and this was not observed. Based on the experimental results, it is likely that the difference in responses between the two assay types is due to batch-to-batch variation in antibody quality (avidity, degree of enzyme labeling and enzyme activity), since different batches of antibody were used in the dual assays compared to the single-analyte assays. The detection limits of the sensors were determined to be 1 and 2 ng/mL for the goat IgG and mouse IgG sensors, respectively (calculated as three times the standard deviation of the background signal). These detection limits are comparable to those of commercially available optical-based single-analyte IgG ELISA kits (e.g. TiterZyme, Assay Designs, Inc., Ann Arbor, MI).

**Simultaneous Immunoassays for the Tumor Markers CEA and AFP.** We are interested in developing electrochemical SMIA sensors for measuring the concentrations of panels of tumor markers. Tumor markers are biomolecules (e.g., proteins, hormones) that are often found in abnormally high amounts in the blood, urine, or tissue of patients with certain types of cancer. Measurement of a single tumor marker often has limited diagnostic value because most markers are not specific and they can have elevated levels in patients without cancer. However, most cancers have more than one marker associated with their incidence, and studies indicate that the diagnostic value of tumor markers may be increased if panels of tumor markers associated with a particular type of cancer are measured. The development of electrochemical SMIA sensors will enable such measurements to be made in a rapid and economical manner. To demonstrate the simultaneous detection of two tumor markers, the colorectal cancer marker, CEA, and the liver and germ cell cancer marker, AFP, were chosen as targets.

In a typical ELISA procedure, one enzyme-labeled antibody is required for each analyte tested. For a sensor designed to detect many analytes, this would require many enzyme-labeled antibodies. Enzyme-labeled antibodies are often expensive and labor-intensive to prepare and purify, and so it would be desirable to reduce the number required for the multianalyte assay. In our two-analyte tumor marker assay, therefore, we used biotin-labeled antibodies, which are significantly less expensive, and easier to prepare and purify, than AP-labeled antibodies. This method also
has the additional advantage that streptavidin-labeled AP can then be used to detect all analytes (Figure 5).

Sensors for the tumor marker immunoassays were prepared by immobilizing anti-CEA on electrode W1 and anti-AFP on electrode W2. To determine the background responses due to nonspecific binding of reagents, the sensors were used in ELISAs with samples of blocking buffer containing no CEA or AFP. Electrodes W1 and W2 produced small background responses of 0.85 (±0.05) and 0.35 (±0.05) μA/cm², respectively. To determine the extent of cross-reaction between CEA and anti-AFP, and between AFP and anti-CEA, sensors were used in ELISAs with samples containing only CEA (1 μg/mL, 200 μL) or only AFP (1 μg/mL, 200 μL). In the assay using samples containing CEA, the anti-CEA electrode (W1) and the anti-AFP electrode (W2) produced current responses of 12.5 (±0.7) and 0.35 (±0.05) μA/cm², respectively. Conversely, in the assay using samples containing only AFP, the anti-CEA electrode (W1) produced only a small response, 0.85 (±0.05) μA/cm², while the anti-AFP electrode (W2) produced a larger current response, 8.9 (±0.7) μA/cm².

These experiments demonstrated several important features: (1) The individual assay systems for CEA and AFP performed as desired; i.e., antibodies immobilized on electrode W1 captured CEA in the sample, and antibodies immobilized on electrode W2 captured AFP in the sample; (2) background signals due to nonspecific binding of reagents were small compared to amperometric signals produced due to specific binding of antibodies; (3) there was no cross-talk between the sensors, since the small background responses observed were produced immediately following addition of HQDP and remained constant throughout the experiments; and (4) there was no significant cross-reactivity between antibodies and nontarget analyte (background responses in the presence of nontarget analyte were similar to those in the absence of analyte). Interestingly, the background response of electrode W1 (the CEA sensor) was consistently higher than that of electrode W2 (the AFP sensor), indicating either a small degree of cross-reactivity between anti-CEA and AP-antibody or a different protein surface coverage of electrode W1 compared to W2, leading to different levels of nonspecific binding of AP-labeled antibodies.

To demonstrate the quantitative simultaneous immunoassays for CEA and AFP, the dose–response behavior of the sensors was determined in ELISAs using samples containing mixtures of CEA and AFP (0–300 ng/mL, 200 μL). In these experiments, the amperometric responses of the anti-CEA electrode (W1) and the anti-AFP electrode (W2) were recorded as a function of CEA and AFP concentrations, respectively. As desired, both electrodes produced amperometric responses that increased as the concentration of complimentary tumor marker increased (Figure 6). The CEA sensor produced a dose–response curve that increased linearly between 25 and 150 ng/mL, with nonlinearity due to the...
onset of sensor saturation being observed above 150 ng/mL. The AFP sensor produced a linear dose–response curve over the entire concentration range studied (10–200 ng/mL). The detection limits of the sensors were determined to be 1 and 1.2 ng/mL for AFP and CEA, respectively. Although these detection limits are higher than those of commercial fluorescence-based CEA and AFP assays (typically 0.2 ng/mL), the immunosensors produced independent dose–response behavior over the physiological concentration ranges associated with the tumor markers.

CONCLUSION

Electrochemical immunosensors have been developed that simultaneously measure the concentrations of two different analytes. This was achieved by using sensors that contained two immunosensing electrodes, with each electrode containing a different immobilized capture antibody. Antibodies were immobilized to the electrodes by covalent attachment to a three-dimensional porous IrOx matrix using silane coupling chemistry. The spatial separation of the electrodes allowed individual immunoassays to be performed at each electrode without interference due to cross-talk. The lack of cross-talk between the electrodes indicates that the sensors can be located significantly closer together than 2.5 mm, which will enable the fabrication of smaller devices. By including additional electrodes in the sensor design, the concept described here can be readily extended to the SMIA of more than two analytes. Sensors for the measurement of goat and mouse IgG, and for the measurement of two tumor markers, CEA and AFP, were demonstrated. The detection limits of the IgG sensors were comparable to commercial optical-based single-analyte IgG assays. The detection limits of the tumor marker sensors were higher than those of commercial optical-based single-analyte CEA and AFP assays. However, the tumor marker assays described here were unoptimized, and increased levels of detection are expected through optimization of sensor fabrication and assay conditions. The work presented here validates the design and concept of our multianalyte electrochemical immunosensor and provides the foundation for the development of smaller detectors with increased multianalyte capability.

ACKNOWLEDGMENT

Financial support for this work was provided by the National Cancer Institute under Grant 1 R43 CA96250-01. The author thanks Dr. Savvas C. Makrides and Dr. R. David Rauh for proofreading this manuscript.

Received for review October 4, 2004. Accepted December 15, 2004.

AC0485278