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AN ULTRASENSITIVE PROTEIN ARRAY BASED ON ELECTROCHEMICAL ENZYME IMMUNOASSAY

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1. ABSTRACT

An ultrasensitive electrochemical enzyme immunoassay (EEIA) for the detection of proteins on an 8x8 array is described. The assay is based on wired enzyme technology. Briefly, capture antibody was covalently immobilized on a self-assembled 11-mercaptoundecanoic acid (MUA) monolayer coated gold electrode. After incubating with a target protein (antigen), the gold electrode was treated sequentially in biotinylated detection antibody solution and in avidin-horseradish peroxidase conjugate (A-HRP) solution. A cationic redox polymer (electrochemical mediator) overcoating was applied to the gold electrode through layer-by-layer electrostatic self-assembly. The formation of a bilayer brought the HRP in electrical contact with the underlying electrode, making the bilayer an electrocatalyst for the reduction of hydrogen peroxide where the redox polymer acts as an artificial mediator. Consequently, the concentration of protein could be quantified amperometrically. This electrochemical immunoassay combined the specificity of the immunological reaction with the sensitivity of the electrochemical detection. The applicability of the system in protein detection was demonstrated with a snake toxin, β-bungarotoxin, a neurotoxin from the venom of the snake *Bungarus multicinctues*. Under optimized experimental conditions, the assay allowed the detection of β-bungarotoxin in the range of 20 pg/mL to1.5 ng/mL with a detection limit of 10 pg/mL (20 fg). A higher detection limit of 25 pg/mL was obtained in serum.

2. INTRODUCTION

The root of protein detection lies in the development of immunoassay. Three approaches, namely, direct immunoassay, (1) antigen capture immunoassays, (2) and sandwich immunoassays, (3) have been proposed. Among them, sandwich immunoassay is the most dominant in protein assays. Both direct immunoassay and antigen capture immunoassay suffer from cross-reaction of antibody with other cellular proteins. Direct immunoassay is not likely to see widespread applications because most interesting proteins are usually in low abundance. They must compete with the abundant proteins for immobilization on the substrate. Moreover, limited selectivity and poor reproducibility of sample preparation and spotting are the other factors limiting the development of direct immunoassays. Unlike antigen capture immunoassay, sandwich immunoassay does not require the analyzed sample to be labeled. This greatly simplifies sample preparation and shorten assay time. The popularity of sandwich immunoassay advanced greatly in the 1970s with the introduction of enzyme-linked immunosorbant assay (ELISA) by Engvall and Perlman.(3) Because of its high sensitivity, excellent selectivity, simplicity and low cost, (3) (4) compared to techniques such as affinity chromatography and mass spectrometry, it has become a standard technology in clinical laboratory.

In the past few years, DNA microarray technology has been adapted for immunoassay-based protein studies. (5) Protein arrays promise to allow high-throughput interrogation of protein activity on a proteomic scale. However, constructing a protein array requires many more steps and more complex technology than that used for DNA microarrays. Unlike DNA microarrays, where high specificities can easily be predetermined by simple chemical synthesis of the capture probe oligonucleotide sequences and high sensitivity can be realized employing a PCR amplification step, the limited availability of proteome-wide bioaffinity reagents has been one of the practical bottlenecks to the protein array approach. Furthermore, to build a viable protein array one has to choose a surface chemistry that will allow immobilized proteins or protein capturing bioaffinity regents to retain their structural and biological

integrities, and to devise an ultrasensitive and versatile means of measuring the degree of protein binding. It has become apparent as in the diabetes homecare market that it is clinically more desirable to develop potable low-density protein array since the need for analyzing a large number of proteins in any one patient is less obvious. The potable devices would enable clinicians to measure a few key proteins at the point of care such as small clinical laboratories and doctors' offices. Optical approaches have been primarily employed in realizing such devices. (6, 7) Although optical detection is very effective for both high and low-density protein arrays, electrochemical detection is more advantageous. The inherent miniaturization of electrochemical biosensors and their compatibility with advanced semiconductor technologies promise to provide a simple, accurate and inexpensive platform for molecular diagnosis. High-density electrochemical protein arrays must substantially lower their cost in order to compete with optical protein arrays. (8) On the other hand, low-density electrochemical protein arrays are more attractive over their optical counterpart. For example, electrochemical measurements can be made on whole blood without interference from blood cells, other proteins, and fat globules. (9) Of the many proposed EEIA, those employing amperometric detections have several distinct advantages over other EEIAs, such as straightforwardness and high sensitivity. Traditionally, amperometric detection is carried in solution phase, relying on a HRP-mediated solution phase reaction for the formation of electroactive solution phase species by means of enzymatically catalyzed oxidation of phenolic compounds. (10, 11, 12, 13) Due to dilution effect, Detection limits were usually at ng/mL levels, which are inferior to ELISA with an optical detection. (4) Various approaches, such as substrate-recycling, (14, 15) the use of rotating-disk electrode, (16, 17) interdigitated array electrode, (17, 18) microfluidic device, (19) and magnetic beads (20) have been proposed to improve the performance of EEIA. For example, a 350-fold amplification of the analytical signal has been achieved using a bienzyme-substrate-recycling scheme. (14)

Several groups, including us, have recently developed ultrasensitive enzyme-amplified amperometric nucleic acid biosensors, which are based on the electrochemical activation of an enzyme tag and monitor the enzymatic reaction through an artificial mediator in the solid phase employing an electrochemical substrate recycling mechanism.(13, 21, 22, 23) Here we show the adaptation of the solid phase electronic transduction in immunoassay to enhance the sensitivity and lower the detection limit of EEIA on an 8x8 array. A protein neurotoxin, β-bungarotoxin (β-BuTx) from the venom of the snake *Bungarus multicinctues*, was chosen as the model analyte. As little as femptograms of β-BuTx (10 pg/mL) was successfully detected. Different from other EEIA previously published, (12, 13) by activating the enzyme tags electrochemically in the bilayer and utilizing the activator as the artificial substrate, the enzymatic reaction can be conveniently monitored amperometrically, and therefore the assay sensitivity and detection limit are markedly improved which are superior to the solution phase EEIA and comparable to the best optical and electrochemical immunoassays.

3. EXPERIMENTAL SECTION

3.1. Materials and apparatus

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich (St Louis, MO) and used without further purification. The redox polymers used in this study were poly(vinylimidazole-*co*-acrylamide), (PVIA-Os) poly(vinylimidazole-*co*-acrylamido-2-methyl-1-propanesulfonic acid), (PVIAMP-Os) poly(vinylimidazole-*co*-acrylic acid), (PVIAA-Os) poly(vinylpyridine-*co*-acrylamide), (PVPA-Os) and poly(vinylpyrridine-*co*-acrylic acid) (PVPAA-Os). Synthesis of the redox polymers was described elsewhere. (24, 25) To demonstrate the "proof of concept", β-BuTx was selected as the model analyte since rabbit polyclonal antibody and monoclonal antibodies (mAb5, mAb11 and mAb15) to this toxin were previously produced and available in our lab. (26) β-BuTx (molecular weight ~8 KDa) was purchased from Sigma (St. Louis, MO, catalogue number T5644). Among the three monoclonal antibodies, mAb15 showed the strongest bioaffinity towards β-BuTx and was therefore selected for this study. The biotinylation of mAb15 was done as described in our previous report. (27) A-HRP was obtained form Sigma.

Electrochemical experiments were carried out using a CH Instruments model 660A electrochemical workstation coupled with a low current module (CH Instruments, Austin, TX). The three-electrode system consisted of a 2-mm-diameter gold working electrode, a miniature Ag/AgCl reference electrode. Phosphate-buffered saline (PBS, pH 7.4), consisting of 0.15 M NaCl and 20 mM phosphate buffer, was used as the supporting electrolyte.

3.2. Protein array fabrication

To fabricate the sensor array, a titanium adhesion layer of 25-50 Å was electron-beam evaporated onto a glass slide followed by 2500-3000 Å of gold. Before antibody modification, the gold coated slide was thoroughly cleaned with freshly prepared piranha solution (98% H_2SO_4/30% H_2O_2 = 3/1. Caution: Piranha solution is a powerful oxidizing agent and reacts violently with organic compounds.) and rinsed with Milli-Q water followed by 10 min in ultrasonic bath in absolute ethanol. The gold surface was then modified immediately after the cleaning step. Initial thiol adsorption was accomplished by immersing the gold substrate in 10 mM MUA in absolute ethanol overnight at room temperature. MUA solutions were freshly prepared before each experiment. The electrodes were rinsed with Milli-Q water and activated with 100 mM of 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide (EDC) and 40 mM of N-hydroxysulfosuccinimide (NHS) in water. A patterned 1-mm thick adhesive spacing/insulating layer with a screen-printed Ag/AgCl layer and a hydrophobic layer were assembled on the top of the slide (Figure 1). The diameter of the individual sensor was 2.0 mm and that of the top hydrophobic pattern was 4 mm. Protein A-purified rabbit

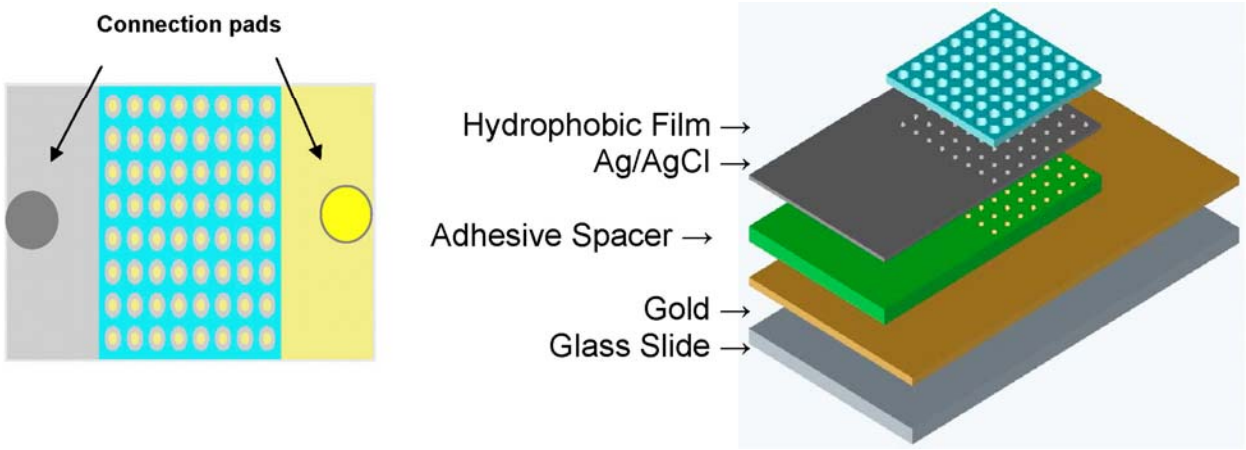


Figure 1. Illustration of electrochemical enzyme innunoassay protein array.

IgG anti-β-BuTx antibody (0.10 mg/mL in PBS) was applied on each of the individual sensor and incubated for 3 h at room temperature. After rinsing with washing buffer (PBS, containing 0.050% Tween-20 (PBS-T)), the unoccupied sites were blocked by incubating with 1.0% bovine serum albumin (BSA) in PBS containing 0.50% Tween 20 overnight at 4 °C. The array was rinsed with washing buffer then stored at 4 °C in PBS solution until used.

3.3. Protein detection

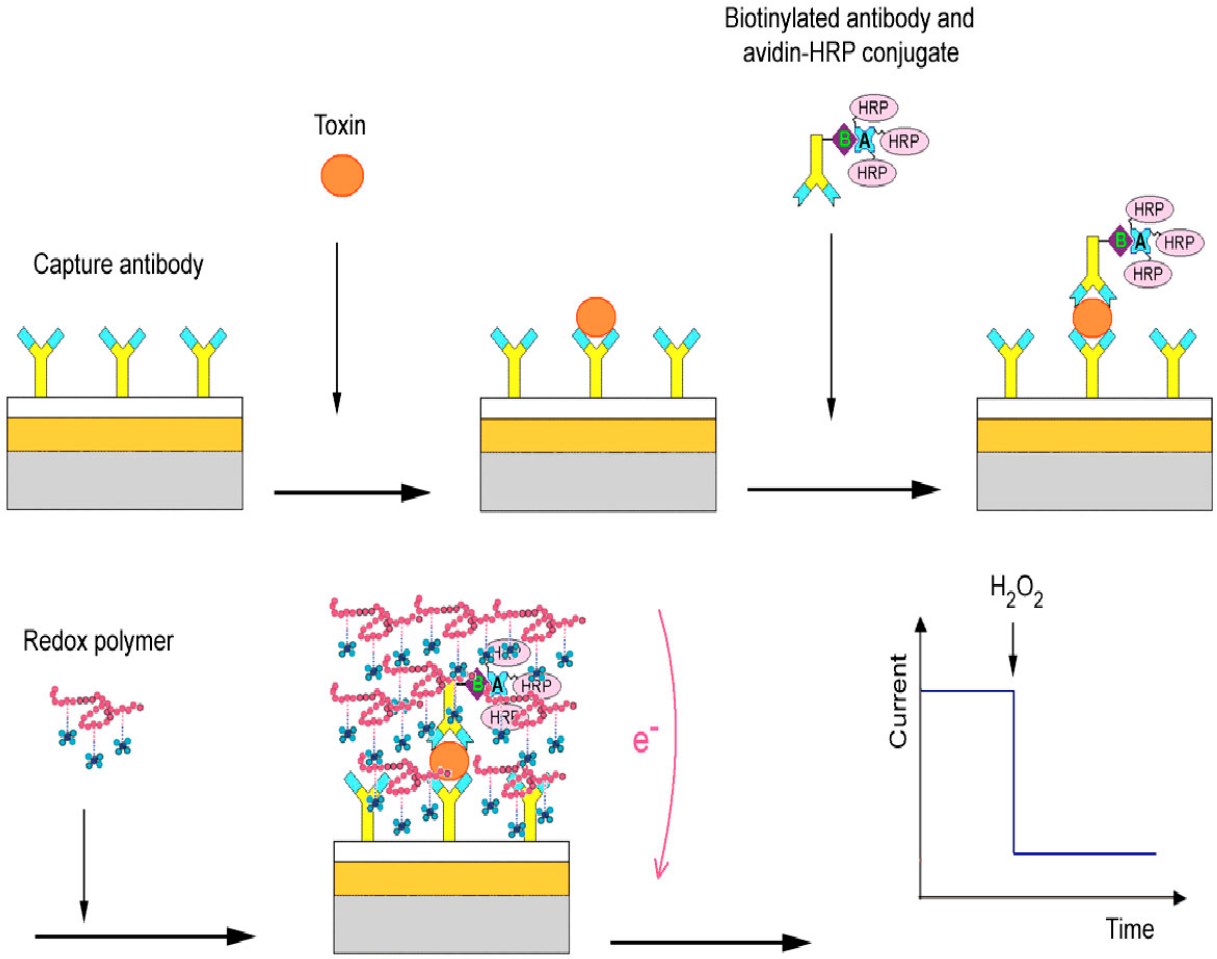
β-BuTx incubation and its electrochemical detection were carried out in five steps, as depicted in Scheme 1. The electrode was placed in a moisture-saturated environmental chamber. Aliquots of β-BuTx solution (2.0 μL) were placed on the sensor and incubated for 30 min. After washing for 10 min in a vigorously stirred PBS solution and drying, biotinylated mAb15 (5.0 μL) was added and the chip was incubated for 30 min. After another washing and drying cycle, A-HRP (5.0 μL) was dispensed onto each chip and incubated for 10 min. The chip was washed, dried and the redox polymer (10 μL) was applied onto the electrode and incubated for 10 min. Electrochemical characterization was carried out with a gold electrode. A Ag/AgCl electrode was used as the reference electrode and a platinum wire as the counter electrode. Detection of β-BuTx was performed on the protein array. The individual sensor remained open-circuit until a 10 μL aliquot of PBS test solution was applied. Withdrawal of the test solution effectively disabled the sensor. The catalytic response was evaluated by amperometry at a constant potential (0.15 V) in PBS containing 2.0-5.0 mM H_2O_2. In the case of lower toxin concentrations, smoothing was applied after each measurement to remove random noise. All incubations and measurement were performed at room temperature. All potentials reported in this work were referred to the Ag/AgCl reference electrode.

4. RESULTS AND DISCUSSION

4.1. Formation of electroactivated bilayer

The fabrication of the protein array for uses in EEIA requires a series of surface chemical reactions. These steps are as follows: (1) formation of a self-assembled monolayer of MUA, (2) reaction of the MUA monolayer with EDC-NHS, (3) covalent attachment of antibody onto the array and (4) treatment of the unreacted sites with a blocking agent, BSA. The fabrication of the array was monitored by different methods such as optical ellipsometric, contact angle, surface coverage and QCM measurements. In step 1, a monolayer of MUA is self-assembled onto the gold substrate. Similar to those reported earlier, all data obtained indicated a single molecular layer of MUA coated on the gold electrode. (28, 29) In step 3, antibody is covalently attached to the surface of the MUA monolayer. The use of chemical coupling approach, instead direct adsorption, has three distinct advantages: (i) It provides much better stability of the immobilized protein, (ii) it avoids the nonspecific adsorption of protein onto the bare gold electrode, and (iii) the surface coverage of the Ab can be manipulated to optimize conditions for protein binding. In the final step of the electrode fabrication, the unreacted sites of the electrode were blocked by reacting with BSA to create a surface that is highly-resistive to nonspecific adsorption of proteins since significant nonspecific adsorption of proteins, particularly A-HRP conjugates, would undoubtedly compromise the accuracy of the monitoring of the protein binding events. BSA is well known for its ability to resist the nonspecific adsorption of proteins, (30) and BSA blocked surfaces are currently used in many protein assays.

PVIA-Os, PVIAMP-Os, PVIAA-Os, PVPA-Os, and PVPAA-Os were first tested for their ability to form stable bilayers. It was found that among these redox polymers, PVPA-Os is the best in terms of stability of the bilayer and the amount of redox polymer being immobilized on the biosensor surface. Apparently, due to partial protonation of acrylamide moieties at pH 7.4, some stability reinforcement is brought to the bilayer, which brings the osmium redox centers in the proximity of A-HRP. Therefore, PVPA-Os was used throughout. As expected, the MUA-antibody monolayer impedes electron transfer between the gold electrode and the solution species No detectable current was observed when tested by cyclic



Scheme 1. Schematic illustration of the bilayer-based electrochemical enzyme immunoassay.

voltammetry in a 0.50 M Na_2SO_4 solution containing 2.5 mM ferricyanide. However, since the redox polymer is positively charged and the electrode is negatively charged, a brief soaking of the electrode in the 5.0 mg/mL PVPA-Os solution, resulted in the formation of an analyte/redox polymer bilayer on the electrode via the layer-by-layer electrostatic self-assembly. (31) As illustrated in Figure 2, the redox polymer coated electrodes exhibited exactly as expected for a highly reversible surface immobilized redox couple. (32) The peak currents were found to be linear with potential scan rate up to 500 mV/s and the ratio of the anodic to the cathodic charge obtained by integrating the current peaks at a very slow scan rates was very close to unit, showing that the charge transfer and counter-ion transfer within the film and the charge transfer from the redox polymer film to the electrode are rapid. A derivation from linearity accompanied by an observable tailing current, occurred when increasing potential scan rate beyond 1.0 V/s. The voltammograms were almost symmetrical at low potential scan rates and the peak-to-peak potential separation (ΔEp) was less than 20 mV. Little change after exhaustive washing with water and PBS and after numerous repetitive potential cycling between –0.2 V and +0.8 V, revealing a highly stable surface immobilized electrostatic bilayer on gold electrode. The presence of HRP in the bilayer did not appreciably alter the electrochemistry of the redox polymer. Later experiments in substrate solution showed that HRP in the bilayer retains its activity. Such results ascertain that all of the osmium redox centers are allowed to reach the electrode surface and proceed to reversible heterogeneous electron transfer. The total amount of bound osmium redox centers, 2.3-6.0x10^{-10} mole/cm^2, depending on the amount of β-BuTx bound to the electrode, was estimated from the area under either the oxidation or the reduction current peak corrected from the background current.

4.2. Feasibility of protein detection

In the first feasibility study, β-BuTx standard solutions were tested on the protein array. Upon applying to the sensor surface at room temperature β-BuTx in the solution was selectively bound by its antibody and immobilized on the sensor surface. Repeated rinsing with PBS completely washed off all the non-immune reaction related β-BuTx. HRP tags were brought to the sensor surface via biotin-avidin interaction during subsequent incubation with the second antibody and A-HRP solution. Typical cyclic voltammograms of the sensor reacted with

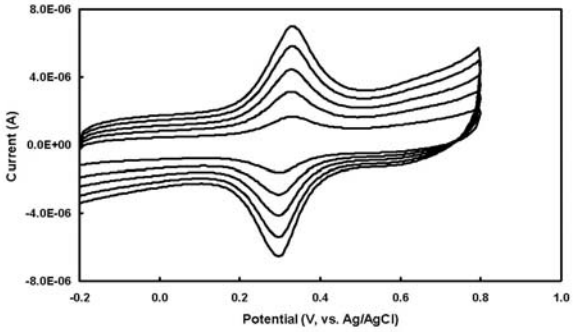


Figure 2. Cyclic voltammograms of a completed sensor at different scan rates in PBS. From innermost to outmost: 100, 200, 300, 400, and 500 mV/s.

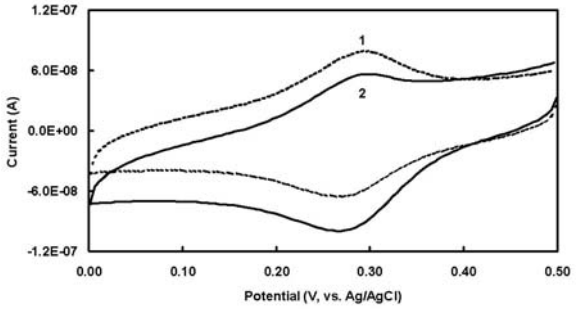


Figure 3. Cyclic voltammograms of sensors at 10 mV/s in PBS (1) after reacting with 2.0 ng/mL β-BuTx, incubation with Ab-biotin, A-HRP, and applying redox polymer overcoating; and (2) after adding 2.0 mM H_2O_2 to the PBS.

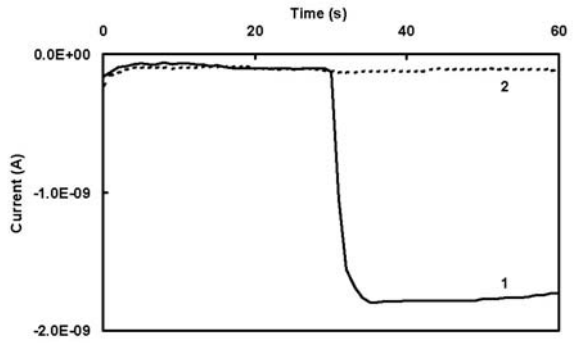
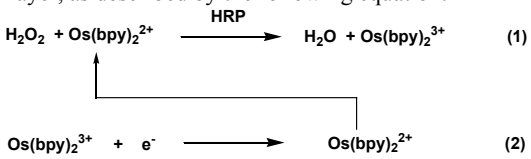


Figure 4. Amperometric responses of (1) Ab coated sensor and (2) BSA coated sensor (control) after reacting with 250 pg/mL β-BuTx, incubation with Ab-biotin, A-HRP and applying redox polymer overcoating. Poised potential: 0.15 V, 2.0 mM H_2O_2.

β-BuTx in PBS (curve a) and in a 2.0 mM H_2O_2 (curve b) are shown in Figure 3. Obvious catalytic current was observed in the presence of H_2O_2 due to the presence of HRP in the bilayer. While in a control experiment, BSA failed to capture any β-BuTx and thereby no enzyme labels were able to bind to the sensor surface. Identical voltammograms were then obtained in PBS and PBS containing H_2O_2 (not shown). No catalytic currents in voltammetry were noticed.

When the completed sensor was immersed in PBS, the reduction current in amperometry increased by 1.8 nA at 0.15 V upon adding 2.0 mM H_2O_2 to PBS (Figure 4). In an identical experiment (control experiment) where BSA was immobilized on the sensor surface, negligible change of current was observed (Figure 4 trace b). The amperometric results complimented the cyclic voltammetric data obtained earlier and confirmed that β-BuTx was successfully detected with high specificity. As expected, the amperometric signal is strongly dependent on the redox polymer loading. The oxidation current increased with increasing the amount of redox polymer up to $2.0x10^{-10}$ mole/cm^2 and then started to level off (Figure 5). It was found that maximal loading of $4.0\text{-}6.0x10^{-10}$ mole/cm^2 could easily be achieved after 5-10 min of adsorption in the 5.0 mg/mL redox polymer solution. To safeguard the amperometric sensitivity, maximal loading was always used for protein detection.

The $Os(bpy)^{2+}$ sites of the redox polymer overcoating effectively activate the HRP tag in the bilayer and acts as the artificial substrate in the enzymatic reduction of H_2O_2. Moreover, at the applied potential of 0.15 V, the thus oxidized redox polymer is instantly reduced, forming a substrate-recycling mechanism in the bilayer, as described by the following equation:



When the reduction potential for Os^{3+} is sufficient, the overall reaction rate and hence the sensitivity of the system is determined by equation (1), or in other words, by the apparent activity of HRP in the bilayer.

To test for possible catalysis by HRP through direct electron-exchange with the substrate electrode, a sensor without applying the redox polymer was fabricated and its voltammogram was measured in PBS containing H_2O_2. Comparison of the voltammetry and amperometry with that of an electrode that was not treated by β-BuTx showed no measurable difference. Furthermore, while H_2O_2 is catalytically electroreduced already at a potential as positive as 0.30 V vs Ag/AgCl on the electrode with the redox polymer overcoating, electroreduction of H_2O_2 was not observed on a gold electrode exposed to the HRP solution or PVPA-Os solution at potentials negative of 0.20 V, ruling out the possibility that the reduction of hydrogen peroxide is catalyzed by immobilized HRP or by PVPA-Os.

4.3. Calibration curve for β-BuTx

Figure 6 shows representative amperometric data obtained from the protein array treated with solutions of increasing concentrations, from 10 pg/mL to 2.5 ng/mL. As the concentration of β-BuTx was increased, the H_2O_2 reduction current increased accordingly in amperometry. The toxin concentrations were proportional to the reduction currents suggesting that the biosensor can be used for quantification purpose. Under optimal experimental conditions, A dynamic range was found to be from 20 pg/mL

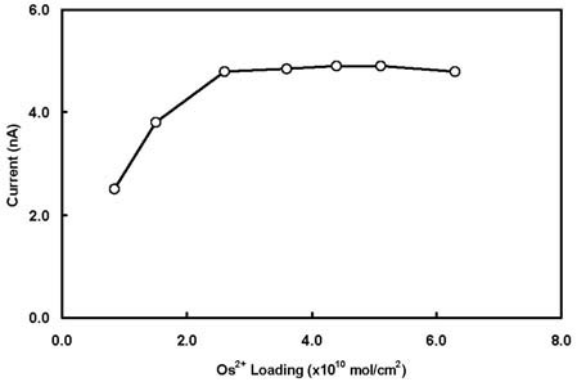


Figure 5. Effect of redox polymer loading on the amperometric response of 1.0 ng/mL β-BuTx. Poised potential: 0.15 V, 2.0 mM H_2O_2.

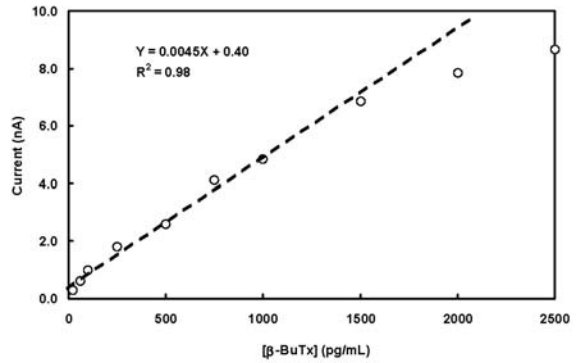


Figure 6. Amperometric response of different β-BuTx concentration in 2.0 μL droplets. Poised potential: 0.15 V, 2.0 mM H_2O_2.

to 1.5 ng/mL with a detection limit of 10 pg/mL estimated from 3-fold of noise. It was found that a practically constant current (saturation current) was observed at a β-BuTx concentration of 20-50 ng/mL. A higher detection limit, 25 pg/mL, was observed when working with serum samples.

5. CONCLUSIONS

It is demonstrated that the use of solid phase EEIA, adapted from the nucleic acid sensor format, offers new possibilities for much improved protein assay. The association of the redox polymer mediator to the HRP tagged antibody led to the electrochemical activation of HRP, generating a measurable current upon adding H_2O_2 to the solution. With the developed EEIA for proteins, a very low detection limit (10 pg/mL of β-BuTx) and a broad linear dynamic range up to 1.5 ng/mL allowed for the development of quantitative assay for proteins. While the concept of solid phase EEIA has been only illustrated using the snake toxin model analyte, it could be easily extended to a wide range of proteins of clinical, biological and environmental significance. The detection limit is greatly improved and it is comparable or better than most optical ELISAs. Efforts to fabricate electrochemical protein arrays for multiplexing and incorporate the array into a handheld electrochemical detector are currently underway.

6. ACKNOWLEDGEMENT

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