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Electrochemical Immunoassay for Detecting Hippuric Acid Based on the Interaction of Osmium-Antigen Conjugate Films with Antibody on Screen Printed Carbon Electrodes

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An electrochemical immunoassay based on osmium-hippuric acid (HA) conjugate films onto the electrode is presented for the detection of urinary HA. This is the first report on the use of the oxidative electropolymerization of 5-amino-1,10-phenanthroline (5-NH_2-phen) for immobilizing an antigen, osmium-conjugated HA. As a redox mediator, $[Os(5\text{-amino-}1,10\text{-phenanthroline})_2(4\text{-aminomethylpyridine-HA})Cl]^{+/2+}$ (Os-phen-HA) was successfully synthesized and electropolymerized onto the screen-printed carbon electrodes (SPCEs). The interaction between osmium-HA conjugate films and antibody-HA (*anti*-HA) was performed by cyclic voltammetry (CV) and differential pulse voltammetry (DPV). The electrical signals were linearly proportional to urinary HA in the range of 0.1-5.0 mg/mL, which is sufficient for use as an immunosensor using a cutoff concentration of 2.0 mg/mL in urine samples. The proposed electrochemical immunoassay method can be extended to various applications for detecting a wide range of different small antigens in the health care area.

Key Words : Electrochemical immunoassay, Hippuric acid, Osmium complex, Electropolymerization

Introduction

Hippuric acid (HA) is a major urinary metabolite in toluene-exposed humans with a molecular weight of 180 Da. Toluene is readily available, and widely used for chemical synthesis, paints, thinners, detergents, adhesives, and as a main solvent in petroleum industries. People chronically exposed to toluene, because of occupation or recreational glue sniffing, have demonstrated anatomical changes in the brain and neurobehavioral impairments.[1-3] The most frequently reported neurobehavioral changes from toluene exposure are related to cognitive function, including memory. Most approaches to detect urinary HA were performed by colorimetric reaction, gas chromatography, or high performance liquid chromatography.[4-7] Although these methods have several advantages, their low specificity, complicated procedures and slowness, requirement of large sample volume, and high costs are limitations to be addressed.

Recent technological progress has facilitated simpler and faster immunoassay using small sample quantity, and several chip-based immunoassay systems have been proposed. However, most of these approaches employed simple optical detection methods, which have some limits in increasing portability for the point-of-care testing of illicit chemicals.[8-11] Most of the early electrochemical immunosensors were basically combined with ELISA methods which were labeled with enzymes and metal nanoparticles on the antibodies. Unlike the optical immunoassay, the electrochemical immunoassay for clinical diagnosis has several advantages such as good selectivity, simple instrumentation, relatively low cost, miniaturization, disposability, and full automation.[12-15] Several researchers have studied the detection of small organic molecules by labeling either antibodies or antigens with metals. Liu *et al.* have demonstrated complex matrices for detecting small organic molecules. J. J. Gooding *et al.* have shown a mixed layer of ferrocene-tethered molecular wire and an oligo(ethylene glycol) component.[16,17]

The objective of the present study was to design a simple electrochemical immunosensor for the detection of a small molecule. We have newly developed a detection method for small organic molecules based on the oxidative electropolymerization of 5-amino-1,10-phenanthroline (5-NH_2-phen).[18] The scheme of these immunoassays is shown in Figure 1. In the presence of *anti*-HA, complexation of the *anti*-HA and Os-phen-HA leads to the attenuated passage of current derived from surface-bound osmium redox probe (Fig. 1(a)). The sensitivity of the immunosensor for detecting free HA is clearly affected by the increment of the osmium current. The general HA detection process using the electrochemical method (Fig. 1(b)) is as follows: (1) the Os-phen-HA was immobilized on the electrode and the mixture of *anti*-HA and free HA in an aqueous solution added onto the electrode, (2) the surface-bound HA and free HA were competitively reacted with *anti*-HA, and (3) after the antigen-antibody reaction, the current from the electrochemically active conjugated-Os was measured finally. In other words, the current increased upon interaction of the antibody and free HA; an event motivated by the presence of the HA in the solution. The detection limit of this immunoassay system was one hundredth of a microgram/mL (> 100 μg/mL). In practice, this immunosensor sensitivity meets the cutoff concentration of 2.0 mg/mL for the direct detection of HA in urine.

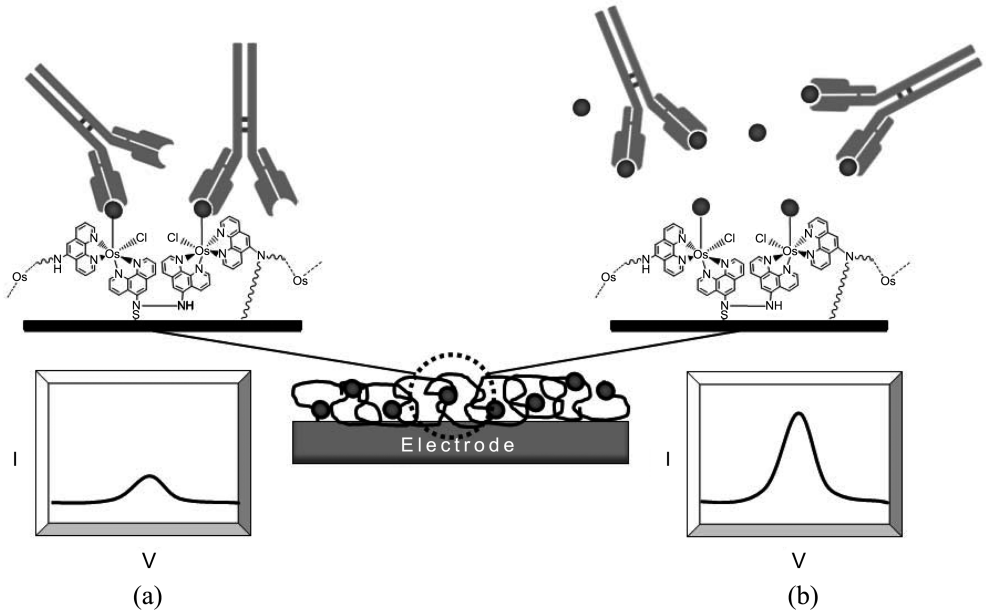

Figure 1. Schematic diagrams of a electrochemical immunoassay. (a) shows the electrode surface before detecting the target antigen with the correlating low electrochemical signal. (b) shows the antibody is combined with the free antigen expected high electrochemical signal.

Experimental

Materials and Reagents. A carbon electrode was screen-printed on OHP film (Electrodag 423SS, Acheson, USA) using a screen printing machine (BS-860AP, Bando, Korea). *N*-ethyl-*N*′-[3-dimethylaminopropyl] carbodiimide (EDC), bovine serum albumin (BSA), *N*-hydroxy succinimide (NHS), hydrochloric acid (HCl), HA, potassium hexachloroosmate (IV), sodium hydrosulfite, ethanol, (1,10)-phenanthroline-5-amino, 4-aminomethylpyridine and sodium hippurate hydrate (NaHA) were purchased from Aldrich (Milwaukee, WI, USA). Monoclonal *anti*-HA was prepared as previously described.[4] The phosphate-buffered saline solution (PBS: 4.3 mM NaH_2PO_4, 15.1 mM Na_2HPO_4, 140 mM NaCl), washing buffer (4.3 mM NaH_2PO_4, 15.1 mM Na_2HPO_4, 500 mM NaCl and 0.5% Tween 20®), and all other solutions were prepared using deionized water (Millipore, Bedford, MA).

Preparation of $[Os(phen)_2(amp\text{-}HA)Cl]^{+/2+}$ (Os-phen-HA). $[Os(phen)_2Cl_2]^{2+/3+}$ was prepared by adapting the previously reported method.[19,20] Typically, 100 mg of K_2OsCl_6 and 88.93 mg of (1,10)-phenanthroline-5-amino (2 equiv) were refluxed in 20 mL of anhydrous ethylene glycol for 1 hour. After cooling the solution, 20 mL of 10 mM sodium hydrosulfite solution was added and the mixture chilled. The resulting red-purple crystals were filtered and washed with ice water. $[Os(phen)_2(amp\text{-}HA)Cl]^{+/2+}$ was prepared by the refluxing 100 mg of $[Os(phen)_2Cl_2]^{2+/3+}$ and 41.3 mg of 4-aminomethylpyridine-HA under N_2 in 20 mL of anhydrous ethylene glycol for 1 hour. After filtering, the resulting solution was added to 2.0 L of anhydrous diethyl ether with vigorously stirring. The dark brown product was precipitated

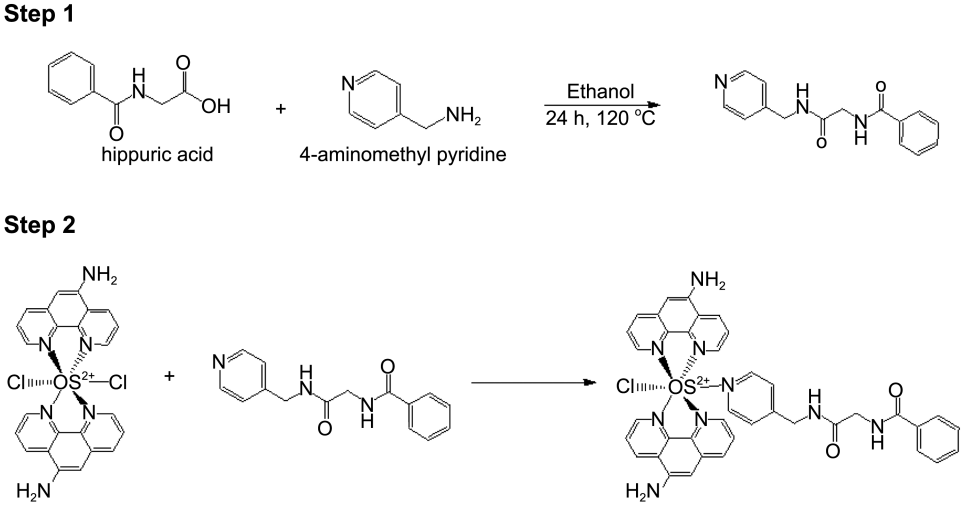

Figure 2. Preparation of a redox complex $[Os(phen)_2(amp\text{-}HA)Cl]$ (Os-phen-HA).

and separated by column chromatography. A cyclic voltammogram of $[Os(phen)_2(amp\text{-}HA)Cl]^{+/2+}$ in PBS buffer (pH 7.2) showed the Os(II/III) wave at 0.35 V *vs*. Ag/AgCl. Validation of Os-phen-HA as an antigen was performed by an immunochromatographic stripe.[4] PBS samples spiked with various concentrations of Os-phen-HA were assayed by immunochromatographic detection device. The analysis was completed within 2 minutes. These results suggested that Os-phen-HA shows a good immunogenic property to *anti*-HA for application in the electrochemical immunoassay.

Electropolymerization of Osmium-HA Conjugate Films on the Electrodes. Screen-printed carbon electrodes (SPCEs) were prepared with Electrodag® 423SS (Acheson, Port Hurton, USA) on OHP film using a semiautomatic screen printing machine. Forty microliter solutions of 2.0 mg/mL $[Os(phen)_2(amp\text{-}HA)Cl]^{+/2+}$ in 0.1 M PBS were loaded onto 3-mm-diameter SPCEs. The supporting electrolyte was 0.14 M NaCl in PBS. The relative rates of polymer film growth could be controlled by applying different cycles from 1 to 50 cycles. The optimum cyclic voltammetry (CV) conditions used for electropolymerization were −0.2 to +1.0 V, with a scan rate of 0.1 V/s for 40 cycles. The morphology of the electropolymerized films at different conditions was investigated using the field emission scanning electron microscopy (FE-SEM) (Fig. 3).

Electrochemical Immunoassay Sensing System. Electrochemical measurements were carried out in a Faraday cage with a CH Instruments model 660A electrochemical workstation (CH Instrument, Austin, TX, USA), interfaced to a computer. The electrochemical characteristics of immobilized Os-phen-HA were studied with the 3.0 mm-diameter working electrodes (SPCEs) on a flexible polyester film. The counter-electrode consisted of 0.5-mm diameter platinum wire and an Ag/AgCl micro-reference electrode (3.0 M KCl saturated with AgCl, Cypress, Lawrence, KS, USA). In order to validate the sensing system, Os-phen-HA electropolymerized electrodes were incubated in the aliquots of monoclonal *anti*-HA with variable concentrations (0.1-10 mg/mL in PBS, pH 7.2) for 5 minutes at room temperature. After the protein complexation, the surface-bound Os redox probes were measured by CV.

For the competitive reaction, 40 μL of the mixture of fixed 2.0 mg/mL *anti*-HA and HA with variable concentrations was introduced by a micropipette onto the electrode. After this reaction, the current was measured by differential pulse voltammetry (DPV). DPV was recorded in PBS (pH 7.2) containing 0.14 M NaCl within the potential range from 0.0 to 0.8V under a modulation amplitude of 50 mV and a scan rate of 100 mV/s with a step potential of 4 mV. The baseline DPVs were corrected by the CHI660A software. Furthermore, in order to prove the feasibility of our detecting system in the presence of human urine media, we conducted "spiked" HA sample analysis, for which the electrode was loaded with 40 μL of mixed solutions (fixed 2.0 mg/mL *anti*-HA and spiked HA in the range of 0-5 mg/mL) in human urine.

Results and Discussion

SEM Characterization of Os-phen-HA Films. Electropolymerized Os-phen-HA films onto SPCEs were observed with SEM (Ed- already defined above). The typical FE-SEM images of the bare SPCEs are shown in Figure 3(a) and those of electropolymerized Os-phen-HA films on SPCEs are shown in Figure 3(b, c, d, e). Repeatedly scanning the potential of SPCEs in 0.1 M PBS with 0.14 M NaCl solution containing 40 μL of 2.0 mg/mL $[Os(phen)_2(amp\text{-}HA)Cl]^{+/2+}$ over −0.2 to +1.0 V *vs*. Ag/AgCl resulted in a continuous

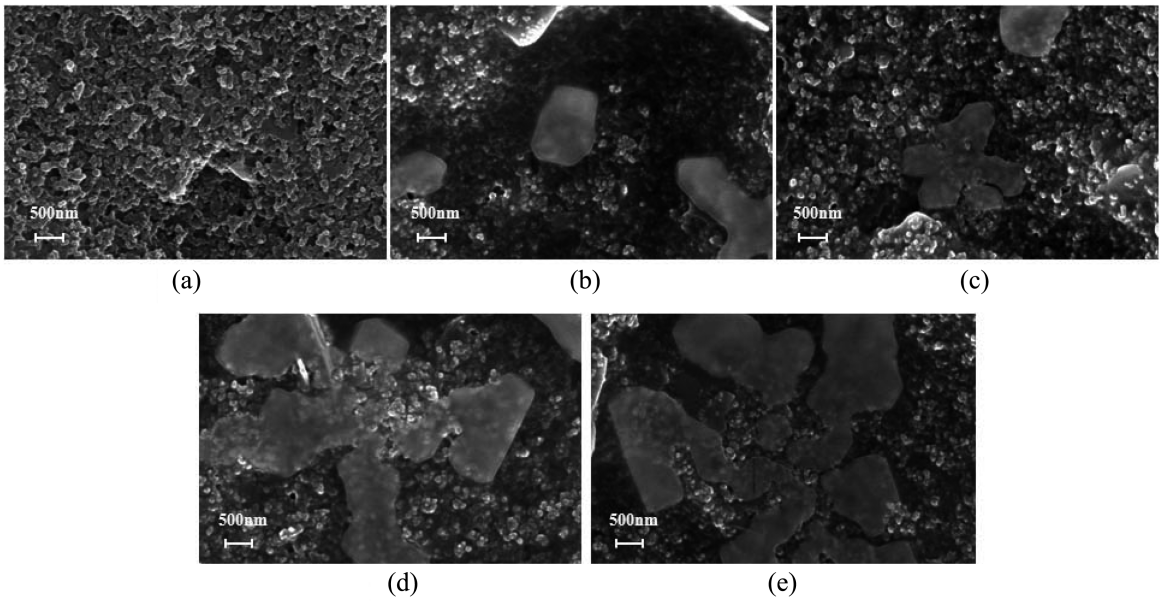

Figure 3. FE-SEM images on bare SPCEs of × 20 K (a), and of Os-phen-HA electropolymerized on a SPCE of × 30 K (b), (c), (d) and (e) from 1.8 mM solution of Os-phen-HA in 100 mM PBS buffer (pH 7.2) with 0.14 M NaCl. The number of cycles were 20 cycles (b), 30 cycles (c), 40 cycles (d) and 50 cycles (e), using the cyclic voltammetric technique.

increase in the size of the polymer films. Wide-area views confirmed that most of the electrode surface was covered by a continuous film, suggesting that nucleation does not influence the film structure. The features of high surface coverage, continuous film formation, and high surface area render these electropolymerized films from ionic liquid potentially useful as sensors and for other applications.[21-23]

Electrochemical Characterization of Os-phen-HA Films. The relative rates of Os-phen-HA film growth can be monitored by CV by applying different cycles and scan rates. As shown in Figure 4, repeatedly cycling of the potential on the SPCEs over the range −0.2 to +1.0 V *vs*. Ag/AgCl resulted in a continuous increase in the area of CV waves in both the cathodic and anodic cycles. Gradually, the peak current associated with the $Os^{2+/3+}$ redox couples increased with increasing number of voltammetric scans. A quasi-reversible redox peak of $Os^{2+/3+}$ couples at +0.340 V versus Ag/AgCl was observed. After a serial of potential scanning, the prepared electrodes were rinsed thoroughly with deionized water and placed in fresh 0.1 M PBS with 0.14 M NaCl solution to obtain the voltammetric wave of $Os^{2+/3+}$. As shown in the inset of Figure 4, the apparent surface coverage (Γ_T^{app}) was determined by the CV waves of $Os^{2+/3+}$ by measuring the charge (Q_c) under the oxidative or reductive wave of the specified metal redox peak and using the following equation.[24,25] In this paper, the calculations of the apparent surface coverage were based on the area of Os $^{+2/3+}$ reductive waves.

$$\Gamma_T^{app} = Q_c/nFA$$

Where n is the number of electrons per molecule reduced ($n = 1$ for the Os-phen-HA studied herein), F represents Faraday's constant, and A is the area of the working electrode in cm^2. The apparent surface coverage by the Os-phen-HA complex increased linearly over 40 cycles, and less thereafter. After 40 cycles, the cycles of the voltammetric waves significantly affected the I_p of $Os^{2+/3+}$. Γ_T^{app} was determined to be $(1.28 \pm 0.25) \times 10^{-9}$ mol/cm^2 (inset in Fig. 4). Hence, a more highly cross-linked polymer containing dispersed osmium redox centers could be obtained by repeatedly cycling of the potential. The exact mechanism of the oxidative electropolymerization of 5-amino-1,10-phenanthroline (5-NH_2-phen) was not elucidated but all evidence to date points to the involvement of a NH radical.[18,26-29] This radical can react with the other amino groups and carbon atoms to form N–N, N=N and N=C polymeric linkers.[23,25]

Immune Reactions of Os-phen-HA and *anti*-HA. Figure 5 shows the attenuation of the DPV response by increasing concentrations of *anti*-HA to an Os-phen-HA electropolymerized electrode in PBS solution. This shows that DPV decreased with each *anti*-HA addition in a well-ordered and predictable trend. Interaction of the antibody immersed the Os-phen-HA in a protein environment and caused a significant attenuation of the cathodic currents derived from the osmium moiety. The attenuation of cathodic currents upon *anti*-HA binding was attributed to the restriction of counter ions accessing the Os-phen-HA to balance the charge, which thereby hindered the electron transfer between electrode and $Os^{2+/3+}$.[30,31] The inset in Figure 5 shows the calibration curves of the cathodic currents at 0.34 V *vs*. Ag/AgCl. As shown in the insets of Figure 5, the detection of cathodic currents gave a non-linear relationship and implies that the kinetics approached saturation at approximately 4.0 mg/mL.

Competitive Immunoassay for HA Analysis. Figure 6 shows typical DPV curves in the competitive immunoassay with Os-phen-HA, in the presence and absence of HA. Following competitive reaction between HA and Os-phen-HA antigen with *anti*-HA, the cathodic signals from Os-phen-HA were directly correlated with HA concentrations. As shown in Figure 6, a high current was observed in the presence of HA. In the presence of HA, high Os-phen-HA peaks result from the competition reaction between HA and Os-phen-HA with *anti*-HA. Combined free HA antigen with *anti*-HA is a heavy molecule with a slow diffusion rate and it

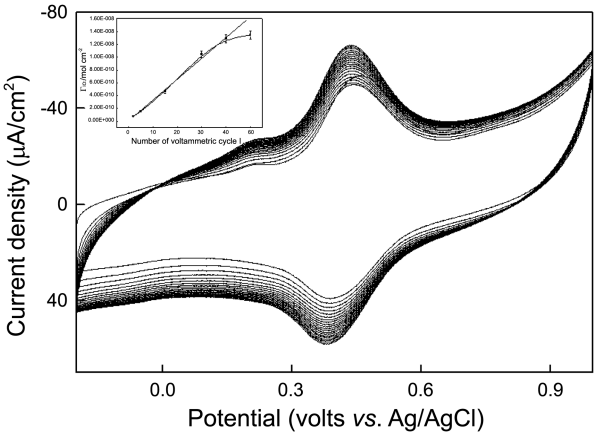

Figure 4. Interfacial electropolymerization from a 2.0 mM solution of Os-phen-HA in 100 mM PBS buffer (pH 7.2) with 0.14 M NaCl at a scan rate of 100 mV/s. Inset: plot of obtained surface coverages versus number of voltammetric cycles for Os-phen-HA films growth (N = 3). N denotes the number of different Os-phen-HA electropolymerized electrodes used.

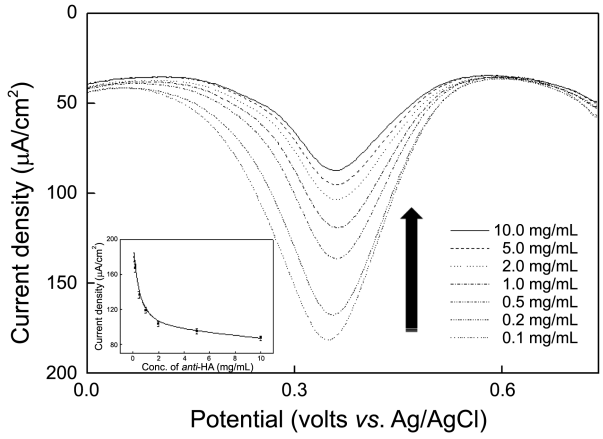

Figure 5. Differential pulse voltammograms of Os-phen-HA films with variable *anti*-HA concentrations from 0.1 mg/mL to 10.0 mg/mL. Reaction conditions: in PBS (pH 7.2, 0.14 M NaCl), at scan rate of 100 mV/s. Inset: DPV cathodic currents at 0.34 V (*vs*. Ag/AgCl) as a function of *anti*-HA concentrations between 0 and 10 mg/mL (N = 5).

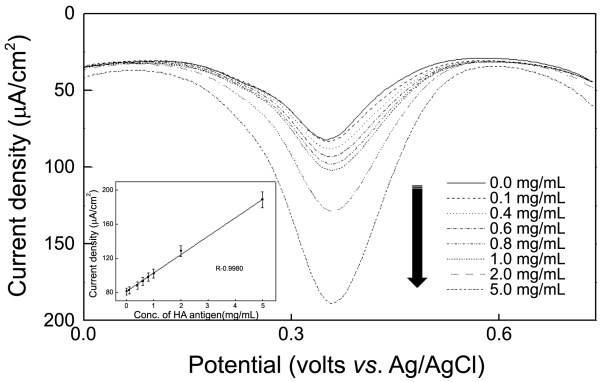

Figure 6. Differential pulse voltammograms of Os-phen-HA films with variable HA antigen concentration from 0.1 mg/mL to 5.0 mg/mL. Reaction conditions: fixed *anti*-HA concentration of 2 mg/mL, in PBS (pH 7.2, 0.14 M NaCl), at scan rate of 100 mV/s. Inset: DPV cathodic currents at 0.34 V (*vs.* Ag/AgCl) as a function of the concentration of hippuric acid between 0 and 5.0 mg/mL. R = 0.9980 (N = 5).

does not obstruct electron transfer of Os-HA antigen on the electrode. In other words, the cathodic current increases in the absence of antibodies from the surface-bounded Os-phen-HA.[32,33]

The insets in Figure 6 show the cathodic current magnitude ($i_{p,a}$) at 0.34 V (*vs*. Ag/AgCl), which was chosen to represent the HA concentration. The detection currents are linear in the HA range of 0.1-5.0 mg/mL, with a correlation coefficient of 0.998. This enabled quantitative analysis of HA using the antibody-binding reaction between HA-antigen and *anti*-HA on the Os-phen-HA electropolymerized electrode with the simple electrochemical immunoassay method.

Real "spiked" HA Analysis in Urine. In order to prove the potential for practical application, we spiked HA into human urine media. Figure 7 shows the cyclic voltammograms of the 1.0 mg/mL Os-phen-HA in the human urine sample. In the pure PBS buffer system, the electrodes showed a quasi-reversible redox response of the Os-phen-HA moieties in Figure 4. In contrast, the human urine sample resulted in a dramatic increase in the anodic current peak at 0.5 V (*vs*. Ag/AgCl). These results show that some of the interfering compounds such as ascorbic acid and uric acid in the human urine sample could be easily oxidized on the electrode.[34-36] However, the interference effects were far removed from the reduction peak of the Os redox probe in Figure 7. The inset of Figure 7 illustrates the peak current magnitude ($i_{p,c}$) at 0.34 V (*vs*. Ag/AgCl), according to the HA concentration (0, 0.1, 0.5, 1.0, 2.0, 4.0, 5.0 mg/mL). The peak current increased linearly with increasing HA concentration with a correlation coefficient of 0.995, which demonstrated the ability of this immunoassay system to detect HA in human urine

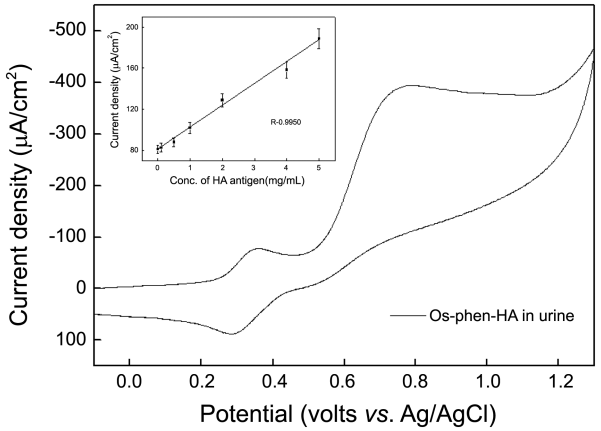

Figure 7. Cyclic voltammogram of Os-phen-HA films in human urine samples at scan rate of 100 mV/s. Inset: DPV cathodic currents at 0.34 V (*vs*. Ag/AgCl) as a function of the concentration of hippuric acid between 0 and 5.0 mg/mL. R = 0.9950 (N = 5).

Conclusions

In conclusion, our immunoassay system was used for the first time in the oxidative electropolymerization of 5-amino-1,10-phenanthroline (5-NH_2-phen) as an electrochemical method for detecting HA. The SEM, CV and DPV results confirmed the successful immobilization of the antigen onto the immune-sensing electrode. The advantages of the presented electrochemical immunoassay method include its short time-detection ability and the simple fabrication protocol of the electrochemical immunoassay. The newly fabricated sensing systems using the osmium-HA conjugate films showed good reproducibility and repeatability in PBS and urine sample. Finally, the proposed electrochemical method can be extended to applications for detecting a wide range of different small toxic molecules in the health care area or in the point-of-care system.

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