Journal of the Korean Electrochemical Society
Vol. 14, No. 1, 2011, 44-49
DOI:10.5229/JKES.2011.14.1.044

Simple Electrochemical Immunosensor for the Detection of Hippuric Acid on the Screen-printed Carbon Electrode Modified Gold Nanoparticles

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(Received January 25, 2011 : Accepted February 21, 2011)

Abstract : This paper describes an electrochemical immunosensor for simple, fast and quantitative detection of a urinary hippuric acid which is one of major biological indicator in toluene-exposed humans. The feature of this electrochemical system for immunoassay of hippuric acid is based on the direct conjugation of ferrocene to a hippuric acid. With the competition between the ferrocene-hippuric acid complex and hippuric acid for binding to the anti-hippuric acid monoclonal antibody coated onto gold nanoparticles, the electrical signals are turned out to be proportional to urinary hippuric acid in the range of 0.01-10 mg/mL, which is enough to be used for the point-of-care. The proposed electrochemical method could extend its applications to detect a wide range of different small molecules of antigens in the health care area.

Keywords : Immunosensor, Hippuric acid, Ferrocene, Point-of-care

Introduction

Hippuric acid (HA) is a major urinary metabolite in toluene-exposed human with a molecular weight of 180 Da. Toluene is readily available, widely used for chemical synthesis, paint, thinner, detergents, adhesive, and petroleum industries as a solvent. People chronically exposed to toluene, because of occupation or recreational glue sniffing, have demonstrated anatomical change in the brain and neurobehavioral impairments [1-3]. The most frequently reported neurobehavioral changes from toluene exposure are related to cognitive function, including memory. Inhaled toluene is mainly metabolized to HA in the liver and secreted in urine.[4] Generally, the urinary HA can be measured by using the rapid immunochromatographic strip (ICS), gas chromatography (GC), or high performance liquid chromatography (HPLC).[4-7] An ICS test, which combines chromatography with conventional immunoassay, is an inexpensive, disposable, membrane-based method that provides visual evidence of the presence of an analyte in a liquid sample. However, an ICS test is not sufficiently sensitive or specific to create a portable point-of-care medical diagnostic system. The visual readout of the strip is usually limited to yes/no answer; this is not adequate when the level of an analyte is important. Therefore, much effort has been directed to develop quantitative ICS tests, which can offer accurate concentration information for the target of interest.

Recent progress of technology facilitates immunoassay simpler and faster. Several chip-based immunoassay systems using small quantity of sample have been proposed. Most of these approaches, however, employ simple optical detection methods, which have some limits on portability for the point-of-care testing of illicit chemicals.[8-11] Most of the early electrochemical immunosensors are basically combined with an ELISA method. The amount of enzymes and metal nanoparticles labeled on antibody is proportional to the currents. 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 fully automation.[12-15]

Most of researchers have studied to label either

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antibody or antigen with metals or enzymes for the detection of small molecules. For example, the ferrocene (Fc)-conjugated IgG for detection of histamine and human chorionic gonadotropin (HCG) has been reported.[16-18] Songqin *et al*. have developed a ferroceneboric acid-based biosensor for glycated hemoglobin. Nam *et al*. have introduced a novel nanoparticle-based biomarker for detection of prostate specific antigen (PSA). Das *et al*. have developed a gold-nanoparticle-based assay for specific proteins. Purushothama and co-workers have reported an alkaline phosphatase-labeled immunosensor system.[19] Zhang and Heller have shown a horseradish peroxidase (HRP)-labeled antibody with the electrode-bounded redox hydrogel polymers.[20]

Previously, the immunochromatographic qualitative detection of HA in urine was reported with the lateral flow system using Anti-HA monoclonal antibody whose cutoff concentration was adjusted to 2 mg of HA/mL of urine.[7] In this paper, we report a simple electrochemical immunosensor to detect HA quantitatively. In our immunoassay systems, HA was directly conjugated to Fc and used without further modifications. The retaining hippuric acid conjugated to ferrocene (HA-Fc conjugate) has shown to interact with anti-HA monoclonal antibody in immunoassay. The electrochemical currents of unbound HA-Fc conjugate can be detected through competitive reaction between free HA and HA-Fc conjugate. The detection limit of our system was at a tenth microgram/mL (> 10 μg/mL). In the practical point of view, this range of sensitivity meets the cutoff for direct detection of hippuric acid in the urine.

Experimental Sections

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-hydroxysuccinimide (NHS), hydrochloric acid, ferrocenemonocarboxaldehyde (Fc-CHO), hippuric acid (HA), hippuric acid-lysine (HA-Lys), and sodium borohydride ($NaBH_4$) were purchased from Aldrich (Milwaukee, WI). The phosphate buffered saline solution (PBS : 4.3 mM NaH_2PO_4, 15.1 mM Na_2HPO_4, 140 mM NaCl), the 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 (Barnstead, Nanopure II, Van Nuys, CA). Monoclonal antibody isotyping Kit I (HRP/ABTS) was purchased from Pierce (Rockford, IL). All other reagents were used without further purification. All solutions were prepared using ultrapure water (18 MΩ, Millipore, USA) or autoclaved water in this work.

Preparation of anti-HA monoclonal antibody and Fc-HA conjugates

The preparation of monoclonal HA-antibody was carried out following a previously papers. HA-antibody was then purified from the ascites with a protein G column affinity chromatographic technique, and isotype, cross-activity, sensitivity, and specificity were analyzed. The conjugation of ferrocene (Fc-CHO) to HA-Lysine was carried out following reported process with a few modifications (Fig. 1).[18,21,22] The synthesis of Fc-HA was verified with thin-layer chromatography (TLC) and ^{1}H NMR spectroscopy.

Electrodeposition of gold nanoparticles on SPCEs

As shown in Fig. 3, screen-printed carbon electrodes (SPCEs) were prepared with Electrodag® 423SS (Acheson, Port Hurton, USA) on OHP film using a semi-automatic screen printing machine. Forty microliters of 1.0 mM $KAuCl_4$ in 0.5 M H_2SO_4 was loaded onto 3-mm-diameter SPCEs. Gold nanoparticles (Au-NP) were electrodeposited by applying a cathodic current at 1.41×10^{-5} A for 300 seconds using the chronopotentiometry technique of a CHI 660B Electrochemical Workstation (CH Instruments, TX, USA).

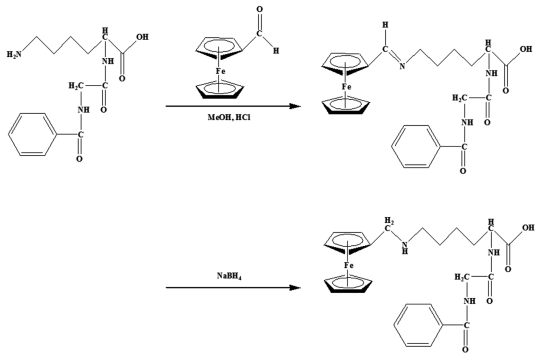

Fig. 1. Preparation of ferrocene-conjugated HA-Lysine (Fc-HA).

Characterization of Au-NPs on SPCEs

Au-NPs electrodeposited onto SPCEs were observed with scanning electron microscopy. The typical FE-SEM images of the bare SPCEs are shown in Fig. 2(a) and those of electrodeposited gold nanoparticles on SPCEs are shown in Fig. 2(b). The average size, shape, and surface density of Au-NP on SPCEs can be controlled by applying different electrochemical deposition times and cathodic current intensities. Finot *et al*. (1999) reported the effects of tetrachloroaurate (III) ion concentrations and deposition potentials on the size of Au nanocrystal. Au-NP grew slowly on a glassy carbon surface during the reduction process of $AuCl_4^-$ ions in acidic solution. However, its average size and surface density increased as electro-deposition time was extended. The Au-NPs growth pattern from our work was similar to that described previously by El-Deab *et al*.[23-25)]

Measurement principle of the electrochemical immunoassay

As shown in Fig. 3, the general principle of the HA immunoassay are as follows: (1) 40 μL of 10 mM 3-mercaptopropionic acid was pipette on the SPCEs, (2) 50 μL of mixture of 5 mg/mL EDC and 1 mg/mL NHS

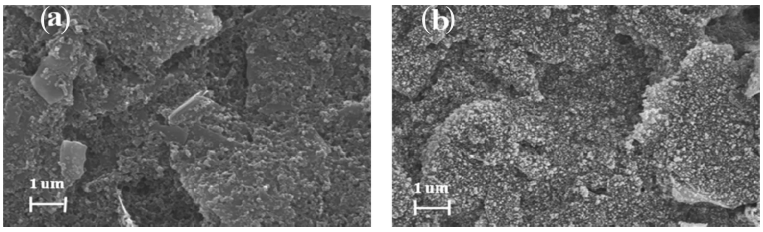

Fig. 2. FE-SEM images on bare SPCEs of × 50 K (a), and those of electrodeposited gold-nanoparticles on SPCE of × 50 K $KAuCl_4$ dissolved in 0.5 M H_2SO_4 solution. Electrodeposition cathodic current was 1.41 × 10^{-5} Å for 300 sec using the chr technique.

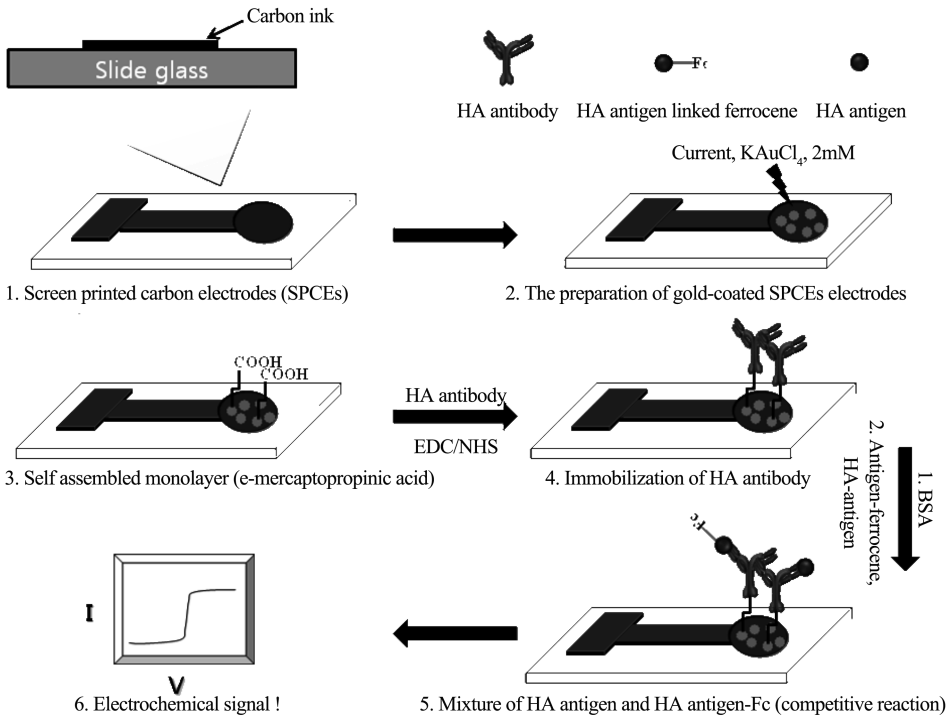

Fig. 3. Schematic diagrams of the reaction of antibody-antigen linked ferrocene complex on the gold nanoparticles printed carbon electrodes (SPCEs).

was added to the electrode, and then the resulting solution incubated for 2 hours at room temperature, (3) 40 μL of 1 mg/mL anti-HA was immobilized with gold nanoparticles on SPCEs, (4) 40 μL of the sample solution (a mixture of fixed 1.0 mg/mL of Fc-HA and HA with variable concentration) was introduced by a micropipette, and both of Fc-HA and HA competed to bind to anti-HA on the gold nanoparticles. After this reaction, the bounded of Fc-HA was measured by the voltammetric (CV, and DPV) techniques. The electrical signals associated with the bounded Fc-HA.

Electrochemical detection

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 Fc-HA were studied with the 3.0 mm-diameter working electrodes (SPCEs) which were made by screen-printing hydrophilic carbon ink (Electrodag® 423SS from Acheson, Port Hurton, MI, USA) on a flexible polyester film. A counter-electrode consists 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).

Results

Validation of Fc-HA as an antigen using an immunochromatographic strip

A schematic description of an immunochromatographic detection device is shown in Fig. 4. In the detection device, the goat anti-mouse IgG and HA-BSA conjugate are separately stripped onto the control (C) and the test lines (T), respectively. The conjugate A (anti-HA antibody-gold colloid conjugate) reacts with goat anti-mouse IgG antibody and can be trapped in the control line regardless of whether a specimen contains HA or not. If a specimen did not contain HA (PBS only), the conjugate A was able to bound to the solid-phase test line coated with HA-BSA conjugate. Therefore, the negative result can be judged by the appearance of two red line signs on the control and test lines. On the other hand, the positive result can be judged by the appearance of only a single line sign on the control line.

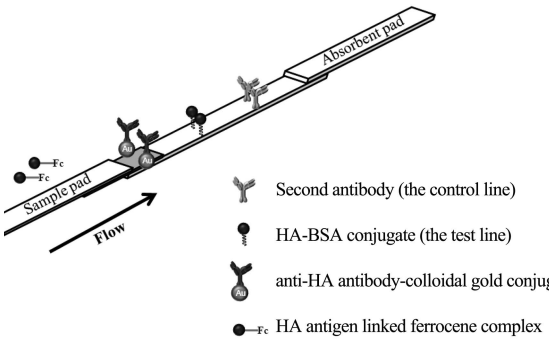

Fig. 4. Design of one-step immunodiagnostic membrane strip in a plastic device for hippuric acid detection. The anti-HA antibody-colloidal gold conjugate and the second antibody do not appear by naked eyes.

Following to the American Conference of Governmental Industrial Hygienists (ACGIH) report, the cutoff concentration of HA in urine was determined to 2 mg/mL. Phosphate buffered saline spiked with various concentrations of Fc-HA were assayed by immunochromatographic detection device (Fig. 5). Analysis was completed within

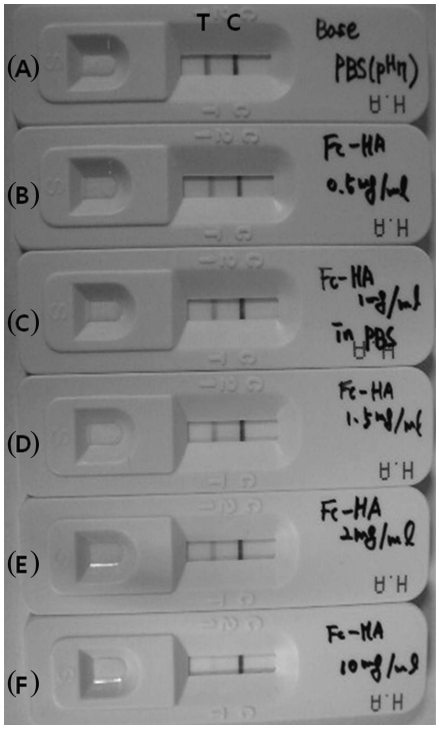

Fig. 5. Immunochromatographic detection of Fc-HA. The phosphate buffered salines spiked with various concentrations of Fc-HA were assayed. Analysis was completed within less than 2 minutes. In the detection zone, the goat anti-mouse IgG and HA-BSA conjugate are separately stripped onto the control line (C) and the test line (T), respectively.

less than 2 minutes. Two red lines were visible in the control and test lines at the lower concentration below 0.5~2 mg/mL of Fc-HA (Fig. 5(B)-(E)), only one red line was shown up just in the test line at the higher concentration above 10 mg of Fc-HA (Fig. 5(F)). These results suggest that Fc-HA shows a good immunogenic property to anti-HA to apply in the electrochemical immunoassay.

Electrochemical detection with HA

The simple electrochemical immunoassay system is shown in Fig. 3. The antigen-antibody reaction of the Fc-HA and Hippuric acid were examined by cyclic voltammetry. Measurements of the antigen-antibody reaction were performed in a solution of fixed 1.0 mg mL^{-1} Fc-HA with the electrode. Fig. 6 shows the typical cyclic voltammograms in the electrochemical immunoassay with Fc-HA in the presence and absence of HA. Following interaction between HA and Fc-HA, and anti-HA, voltammetric signals from Fc-HA were directly correlated with HA concentrations. As shown in Fig. 6, a very high current was observed in the absence of HA. This electric signal results from an antigen-antibody interaction between Fc-HA and anti-HA-coated on the gold electrode. In the presence of HA, low Fc-HA peaks result from competition reaction between HA and Fc-HA with antibody-HA on the gold electrode. As shown in the inset of Fig. 6, the current magnitude ($i_{p,a}$) at 0.30 V (vs. Ag/AgCl) was chosen to represent the concentration of HA. The peak current decreased linearly with HA concentration in the 1.0-10 mg/mL range. Next, the relative concentrations of HA and Fc-HA were varied. Measurements in the HA range of 1.0-10 mg/mL were performed in a solution of fixed 1.0 mg/mL of Fc-HA with a gold electrode. Inset shows the calibration curves at the gold electrode with HA at different concentrations. The detection currents are linear in the HA range of 1.0-10 mg/mL with a correlation coefficient of 0.998. Therefore, quantitative analysis of HA was possible, using the antibody-binding competition between HA and Fc-HA with the simple electrochemical immunoassay method.

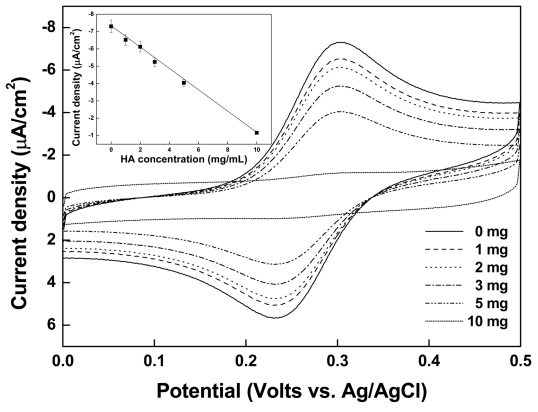

Fig. 6. Cyclic voltammograms of 1.0 mg/mL Fc-HA with variable HA (dash line) and without HA (solid line) at scan rate of 100 mV/s. Inset: peak currents as a function of the concentration of hippuric acid between 0 and 10 mg/mL. R = 0.998 (N = 5). N denotes the number of different SPCEs used.

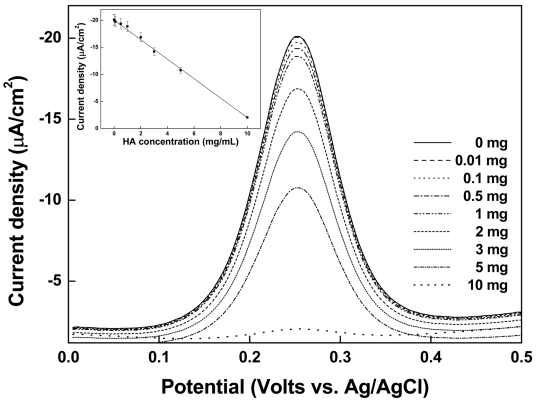

Fig. 7. Differential pulse voltammograms of 1.0 mg/mL Fc-HA with (dash line) and without (solid line) HA at scan rate of 100 mV/s. Inset: peak currents as a function of the concentration of hippuric acid between 0.01 and 10 mg/mL. R = 0.998 (N = 5). N denotes the number of different SPCEs used.

Fig. 7 shows the typical DPV of Fc-HA in the presence and absence of HA. It can be seen that well-defined ferrocene peak was observed in the absence of HA. As shown in the inset of Fig. 7, the current magnitude ($i_{p,a}$) at 0.25 V (vs. Ag/AgCl) was chosen to represent the concentration of HA. The peak current decreased linearly with HA concentration in the 0.01-10 mg/mL range. This result indicates that DPV method is more sensitive than CV.

Conclusions

The electrochemical immunoassay system to detect HA in urine was developed, evaluated, and turned out to be feasible for a portable point-of-care medical diagnostic

system. The advantages of the proposed system include its short time-detection ability and a simple, inexpensive, and disposable membrane-based fabrication protocol of chip without requirement of expensive instruments. Finally, the proposed electrochemical immunoassay method can extend its application to detect a wide range of different small antigens in the health care areas.

Acknowledgement

The present research was supported by the research fund of Dankook University in 2008.

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