Microfluidic chip-based electrochemical immunoassay for hippuric acid

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Urinary hippuric acid (HA), of molecular weight 180 Da, is one of the major metabolites in toluene-exposed humans and is a major biological indicator. Simple and ubiquitous monitoring of exposure to toluene is very important in occupational health care, and a microfluidic chip-based electrochemical immunoassay for rapid and quantitative detection of HA in human urine is proposed in this paper. The system employs a conjugate of ferrocene (Fc) and hippuric acid (HA). The competition between hippuric acid (HA) and the ferrocene-hippuric acid complex (Fc-Lys-HA) to bind with a HA antibody coated onto polybeads generated electrical signals proportional to the HA concentration in the range of 0–40 mg mL^{-1}. All the complicated HA detection processes were integrated on the single microfluidic platform. The quantitative advantages of our HA detection chip are as follows: (1) the total chip size was reduced to 3.0 × 2.0 × 0.5 cm and is small enough to be portable, (2) the assay time took 1 min, and is shorter than that of conventional electrochemical HA immunoassay systems (about 20 min) and (3) 40 μL of the sample solution was enough to detect HA in the range of 0–40 mg mL^{-1}, which is enough range to be used for the point-of-care system. In addition, we suggest the improved chip-based HA assay method by the combination of electrochemical and enzymatic amplification processes for the detection of greater electrical signals. The sensitivity of the combined method was increased about three times compared to that of the non-enzymatic process.

Introduction

Detection of harmful materials with low-molecular weight is very important in the public health and safety. One of the representative materials is toluene, which is a broadly applied compound in chemical synthesis, in paints, in detergents, in adhesives, and in the petroleum industry. Those who are exposed to toluene for long times have been found to suffer from anatomical changes in the brain and neurobehavioral impairments.[1–3] Such neurobehavioral changes are related to the cognitive functions including memory, and simple and ubiquitous monitoring of exposure to toluene is very important in occupational health care. Urinary hippuric acid (HA), of molecular weight 180 Da, is one of the major metabolites in toluene-exposed humans and is a major biological indicator.[4–6] Generally, urinary HA is measured by a colorimetric reaction, gas chromatography (GC), or high performance liquid chromatography (HPLC).[7–10] Although these methods have several advantages, low specificity, complicated procedures and slowness, the requirement of large sample volumes, and high costs are limitations to be addressed.

Recent progress in microfluidic technology facilitates simpler and faster immunoassays using a small quantity of sample, and several chip-based immunoassay systems have been proposed.[11–16] However, most of these approaches employ simple optical detection methods,[17–20] which have some limits in increasing portability for the point-of-care testing of illicit chemicals. In contrast, an electrochemical system has several advantages overcoming the limits of optical detection such as simple instrumentation, relatively low cost, miniaturization, portability, disposability, and full automation.[21–24]

In this paper, we report a new electrochemical immunoassay method for the detection of HA by using a microfluidic chip with an integrated microelectrode. For the electrochemical immunosensing, most researchers have labeled the antibody or antigen with either metals or enzymes. Aguilar and colleagues reported a self-contained microchemical enzyme-linked immunosorbent assay (ELISA) device.[25] Lim and Matsunaga used ferrocene-conjugated IgG for the detection of histamine and human chorionic gonadotropin.[26–28] Wang and co-workers introduced a novel quantum-dot-based biomarker for the detection of prostate specific antigens.[29,30] Purushothama and co-workers have reported an alkaline phosphatase-labeled immunosensor system.[31] Zhang and Heller used a horseradish peroxidase-labeled antibody with electrode-bound redox hydrogel polymers.[32]

Although these approaches are promising, most of them are adapted to the high sensitive detection of materials with low concentration. Therefore, they are not suitable to use in the detection of urinary HA because the HA concentration in urine is relatively high ($\gg 1$ μg mL^{-1}). To address this limitation, we proposed the new approach of a single metal complex attached to each single small antigen. For the simple and portable immunoassay, we employed microfluidic technology. The general HA detection process using the electrochemical method (top of Fig. 1) is as follows: (1) *anti*-HA was immobilized on polybeads

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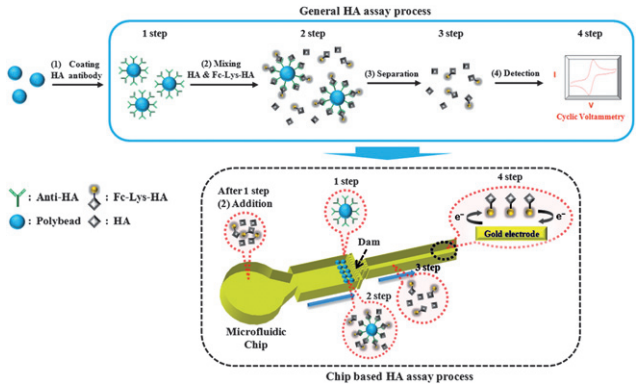

Fig. 1 Schematic diagram of the microfluidic chip-based immunoassay system. HA and ferrocene-conjugated HA-Lysine (Fc-Lys-HA) are mixed well and then injected to the microfluidic channel. The two components are competitively trapped by *anti*-HA antibody-coated polybeads. Following interaction between antigens (HA and Fc-Lys-HA) and antibody (*anti*-HA), the eluted Fc-Lys-HA passes to the electrode. The amperometric signals from Fc-Lys-HA are directly correlated with HA concentrations.

and the mixture of Fc-Lys-HA and HA in an aqueous solution added to polybeads in the tube, (2) the Fc-Lys-HA and HA mixture was reacted with *anti*-HA-bound bead competing for binding, (3) after the competition reaction, the residual of Fc-Lys-HA and HA were separated using a centrifuge and the current from the electrochemically active conjugated-Fc was measured finally.

The general HA detection process is complicated and requires lots of labor, devices and time. We integrated all these complex processes in the single microfluidic platform (Fig. 1 (lower part)) and optimized the detection process. By the chip-based immunoassay, the consumption of labor, sample volume and processing time was greatly reduced. Furthermore, we suggest the method to enhance the detection sensitivity by employing an enzymatic amplification process and a more than 3 times higher current was measured comparing with the current of the non-amplification process.

Experimental

Materials

Polystyrene beads (90 μm) (Catalog no. 07315) were from Polyscience Inc. Polydimethylsiloxane (PDMS, Sylgard 184) was from Dow Corning. NANOTM SU-8(50) and NANOTM SU-8(100) were products of MicroChem (Newton, MA). AZ 5214-E was obtained from AZ Electronic Materials Corporation (Branchburg, NJ). *N*-ethyl-*N*′-[3-dimethylaminopropyl] carbodiimide (EDC), bovine serum albumin (BSA), *N*-hydroxy succinimide (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). Glucose oxidase (GOx) and glucose were purchased from Sigma (St. Louis, MO). Monoclonal antibody isotyping Kit I (HRP/ABTS) was purchased from Pierce (Rockford, IL). All analytical reagents were used without further purification. All solutions were prepared using Milli-Q ultrapure water system (Millipore, Bedford, MA).

Concept of microfluidic chip-based electrochemical immunoassay

The general principle of the HA immunoassay is shown in Fig. 1. First, *anti*-HA is immobilized on polybeads and the mixture of Fc-Lys-HA and HA in an aqueous solution added to the polybead. Second, the Fc-Lys-HA and HA mixture is reacted with *anti*-HA-bound bead competing for binding. After this reaction, the residual of Fc-Lys-HA and HA are separated using a centrifuge and the current from the electrochemically active conjugated-Fc is measured finally.

Here, all these complicated processes were integrated on the single microfluidic platform as illustrated in Fig. 1. 40 μL of the *anti*-HA-bound beads were introduced with a micropipette (20 ± 2 beads/μL) and trapped at the polybead reserving area. Next, 40 μL of the sample solution (a mixture of fixed 1.0 mg mL^{-1} of Fc-Lys-HA and HA with variable concentration) was introduced by syringe for 1 min, and both of Fc-Lys-HA and HA competed to bind to *anti*-HA on the beads. After a 1 min reaction, the unreacted Fc-Lys-HA and HA were leaked out through the dam which plays the equivalent role of a centrifuge, and passed the gold electrode. Then, the current associated with the unreacted Fc-Lys-HA was measured by cyclic voltammetry.

Preparation of HA-BSA carrier protein conjugates

HA was directly conjugated to BSA using EDC and NHS[33,34] as follows: (1) 10 mg of HA was dissolved in 5 mL of 0.1 M phosphate buffer (PBS), pH 5.0, with 30 mg of EDC and 5 mg of NHS, (2) 10 mg of BSA was added to the 5 mL of solution containing HA, and then the resulting solution incubated for 2 h, and (3) the resulting BSA-HA was purified by dialysis against three changes of fresh PBS buffer, pH 7.0.

Preparation of monoclonal HA–antibody conjugates

BALB/c mice were immunized every two weeks with HA-BSA conjugate emulsified with complete or incomplete Freund's adjuvant. After the second immunization, serum samples were obtained and *anti*-HA titers measured using ELISA on a microplate coated with HA-BSA conjugate. When the titer of antibody level in serum was high enough, the mice were finally boosted *via* the tail vein with HA-BSA conjugate without adjuvant. The spleen cells from the immunized mice were then fused with myeloma cell, SP2/0. The freshly harvested spleen cells and SP2/0 cells in PEG were centrifuged and then distributed to 96-well plates containing feeder cells derived from saline peritoneal washes of other mice. During weeks 1–3 after the cell fusion, the titer of *anti*-HA antibody was monitored from the culture media. Anti-HA monoclonal antibody was then purified from the ascites with a protein G column affinity chromatographic technique, and isotype, cross-activity, sensitivity, and specificity were analyzed.

Preparation of ferrocene-conjugated HA-Lysine (Fc-Lys-HA)

The conjugation of ferrocene (Fc–CHO) to HA-Lysine was carried out following a reported process with a few modifications.[28,35,36] As shown in Fig. 2, Fc–CHO was dissolved in 10 mL of 0.5 mmol methanol containing hydrochloric acid, at 70 °C, and then incubated with 15 mg of sodium borohydride ($NaBH_4$)

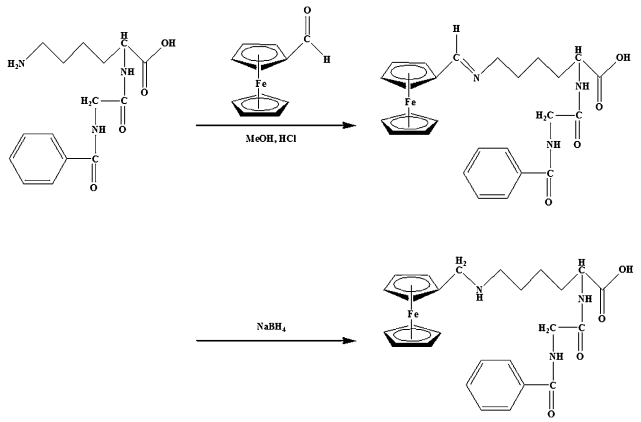

Fig. 2 Preparation of ferrocene-conjugated HA-Lysine (Fc-Lys-HA).

for 2 h. The conjugation of Fc-Lys-HA was verified by thin-layer chromatography (TLC) and ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum (400 MHz, CD_3OD, TMS) showed: δ_{ppm} 7.95–7.45 (m, 5H, phenyl), 4.7–4.2 (m, 9H, Cp), 4.0–4.1 (t, 2H, *J* 7.0 Hz, –C*H*$_2$NH–), 2.55–1.30 (m, 8H, –CH_2–).

Fabrication of the microfluidic immunoassay chip

A schematic of the electrochemical immunoassay chip for HA is illustrated in Fig. 3(a); the system consists of four layers. On the glass slide, a metal electrode layer was deposited and patterned by chemical etching process and a PDMS microfluidic channel was bonded on this glass. The channel has an inlet, an outlet, a polybead reserving chamber (PRC), a detection chamber, and a dam. The PRC is used to store *anti*-HA antibody coated beads and the dam located at the end of the reserving chamber prevents bead spillovers. Fig. 3(b) is a detailed schematic of the PRC and the inset illustrates the micrograph of polybeads in the PRC; the detection chamber is used to measure the current created by unreacted Fc-Lys-HA. Fig. 3(c) is a photograph of the completed immunoassay chip. Microchannels were fabricated using PDMS, as reported previously.[37] Microelectrodes were created by e-beam evaporation of a titanium layer (100 Å in thickness), and a gold layer (1000 Å in thickness) on the glass slide, followed by chemical etching {Au etchant: aqua regia [1 : 3 (v/v) of HNO_3: HCl], Ti etchant [1 : 2 : 7 (v/v) of HF: NO_3: H_2O]}.[38] The thickness of the microchannel for HA delivery was 100 μm and the thickness of the PRC 500 μm. To bond the metal-patterned glass slide (with the electrodes) to the PDMS-based microchannel, both slide and channel were exposed to oxygen plasma using the reactive ion etching (RIE) system, and the bonded microfluidic chip was thermally cured on a hotplate for 2 h at 80 °C.

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-Lys-HA were studied with the 3.0 mm-diameter working electrodes (SPEs) 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). The electrochemical immunoassay in the microfluidic channel was conducted with a patterned gold working electrode (3.5 mm^2), a patterned gold counter electrode (≈ 2.5 mm^2), and a printed Ag/AgCl reference electrode (≈1.0 mm^2) (C61003P7,

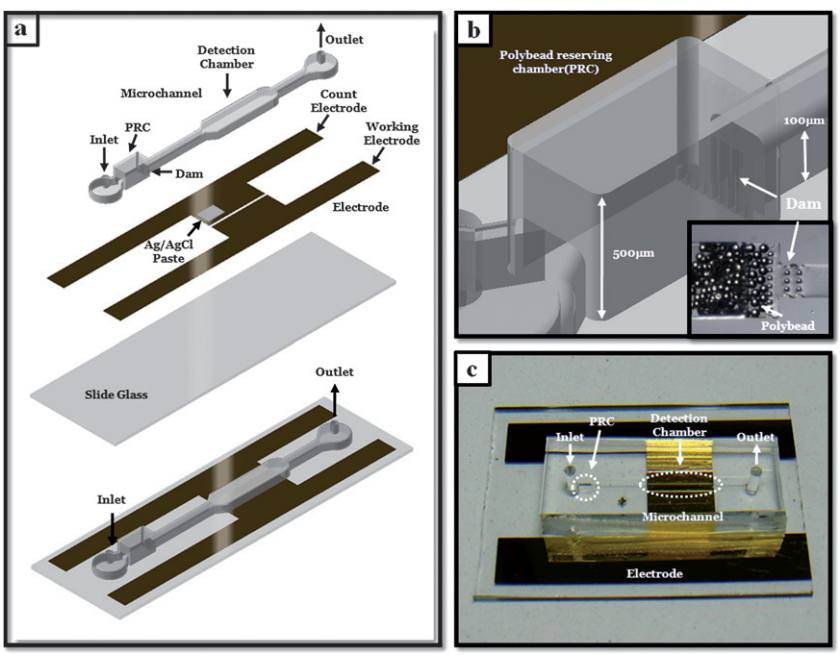

Fig. 3 A schematic 3D diagram of the immunoassay chip. (a) The elements of the immunoassay chip and the assembled immunoassay chip. (b) The chamber holding the polybeads [a magnification of a part of 3(a)]. Inset: A photograph of the polybead reserving chamber (PRC). (c) A photograph of the immunoassay microchip.

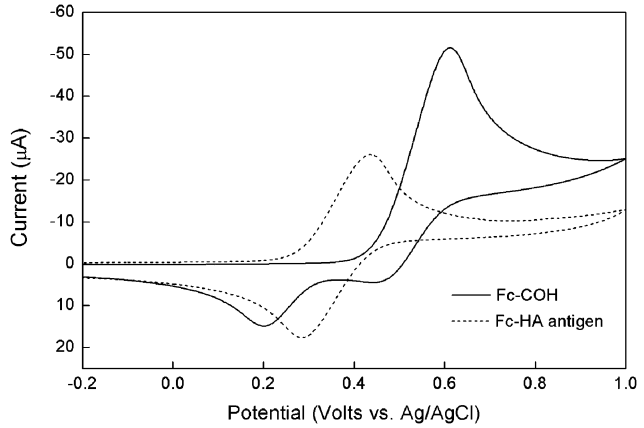

Fig. 4 Cyclic voltammograms of 1.0 mg mL^{-1} Fc-Lys-HA and 1.0 mg mL^{-1} Fc–CHO dissolved in 0.1 M PBS buffer (pH 7.2) with 0.14 M NaCl at a scan rate of 100 mV s^{-1} using a screen printed carbon electrode 3.0 mm in diameter.

Gwent, Torfaen, UK). The Ag/AgCl paste was used for the reference electrode and detail positions of reference, count and working electrodes are noted at Fig. 3(a). For the enzymatic signal amplification, the microfluidic channel was filled with 20 μL of mixed solution (1.0 mg mL^{-1} Fc-Lys-HA and HA in the range of 0–40 mg mL^{-1}), 10 μL of 1.0 mg mL^{-1} of GOx, and 10 μL of 5 mM of glucose in 0.1 M PBS buffer solution. In order to prove that our method is feasible in the presence of a human urine matrix, we carried out "spiked" HA sample analysis. To this end, the microfluidic channel was filled with 40 μL of mixed solutions (1.0 mg mL^{-1} Fc-Lys-HA and spiked HA in the range of 0–30 mg mL^{-1}) in human urine.

Results

Electrochemical characteristics of Fc-Lys-HA

Fig. 4 shows the typical cyclic voltammograms of Fc derivatives. When the cyclic voltammetry of 1.0 mg mL^{-1} Fc–CHO was carried out in 0.1 M PBS buffer (pH 7.2), a quasi-reversible oxidation peak at 600 mV (*vs.* Ag/AgCl) and two irreversible reduction peaks at 400 mV and 200 mV (*vs.* Ag/AgCl) were observed. Similar electrochemical behavior has been previously reported.[35] Following conjugation of Fc–CHO with HA, Fc-Lys-HA showed quasi-reversible oxidation and reduction peaks at $E_{1/2}$ = 350 mV (*vs.* Ag/AgCl).[35,39] These results suggested that Fc-Lys-HA was a fast, quasi-reversible redox mediator that could be utilized for the electrochemical immunoassay of HA.[40]

In Fig. 5, steady-state cyclic voltammograms with 1.0 mg mL^{-1} of Fc-Lys-HA in the microfluidic channel are shown as a function of scan rate. Insets in the Fig. 5 show the oxidation peak currents $[(E_p)_a]$ and the reduction peak currents $[(E_p)_c]$ of Fc-Lys-HA increased linearly with the square root of scan rates ($\nu^{1/2}$) in the range of 10–100 mV s^{-1}. This suggests that the electron transfer process on the electrode is controlled by the diffusion reaction. The value of $E^{\circ\prime}$ for Fc-Lys-HA (360 mV *vs.* Ag/AgCl) was determined by extrapolating the intercept $E_{0.85}$ at $\nu^{1/2} = 0$.[41] Due to the uncompensated resistance of the electrode in the microfluidic channel, the peak splittings $[\Delta E_p = (E_p)_a - (E_p)_c]$ of Fc-Lys-HA in the CV response are gradually increased at fast scan rates.[42]

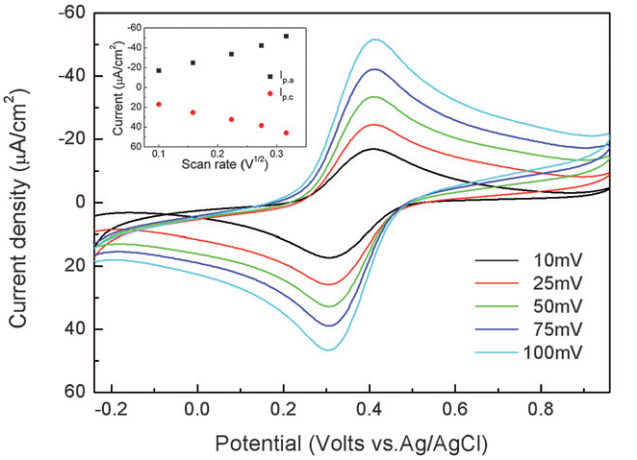

Fig. 5 Cyclic voltammograms of 1.0 mg mL^{-1} Fc-Lys-HA dissolved in 0.1 M PBS buffer (pH 7.2), in the microfluidic channel, at different scan rates (10, 25, 50, 75, 100 mV s^{-1}) using a patterned gold electrode. Inset: The variation of peak currents density *vs.* square root of scan rate. R_1 = 0.985 (black square), R_2 = 0.991 (red dot).

Electrochemical detection of HA

Fig. 6 shows typical cyclic voltammograms in the microfluidic immunoassay with Fc-Lys-HA, in the presence and absence of HA. Following interaction between HA and Fc-Lys-HA, and *anti*-HA, amperometric signals from Fc-Lys-HA were directly correlated with HA concentrations. As shown in Fig. 6, a low current was observed in the absence of HA. This electric signal results from a free interaction between Fc-Lys-HA and *anti*-HA-coated polybeads before introduction onto the electrode. In the presence of HA, high Fc-Lys-HA peaks result from the competition reaction between HA and Fc-Lys-HA with antibody-HA on the polybeads. As shown in the inset of Fig. 6, the current magnitude ($i_{p,a}$) at 0.40 V (*vs.* Ag/AgCl) was chosen to represent the concentration of HA. The peak current increased linearly with HA concentration in the 0–40 mg mL^{-1} range. Next, the

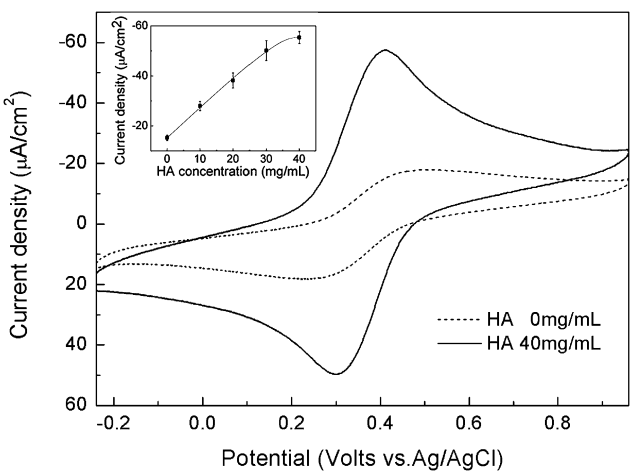

Fig. 6 Cyclic voltammograms of 1.0 mg mL^{-1} Fc-Lys-HA with (solid line) and without (dash line) HA at scan rate of 100 mV s^{-1}. Inset: peak currents as a function of the concentration of HA between 0 and 30 mg mL^{-1}. R = 0.994 (N = 5). N denotes the number of different microfluidic chips used.

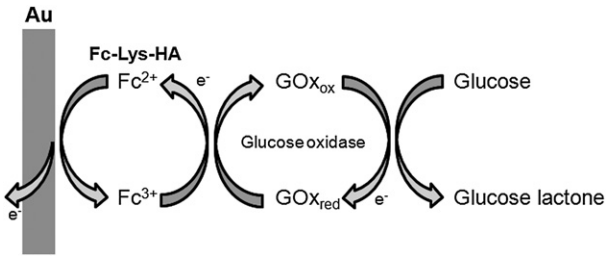

Fig. 7 Steps of catalytic electron transfer in the electrooxidation of glucose to glucose lactone in the presence of GOx and Fc-Lys-HA.

relative concentrations of HA and Fc-Lys-HA were varied. Measurements in the HA range of 0–40 mg mL^{-1} were performed in a solution of 1.0 mg mL^{-1} of Fc-Lys-HA with a gold electrode. The inset shows the calibration curves at the gold electrode with HA at different concentrations. The detection currents are linear in the HA range of 0–30 mg mL^{-1} with a correlation coefficient of 0.994. Therefore, the quantitative analysis of HA was possible using the antibody-binding competition between HA and Fc-Lys-HA in the microfluidic chip.

Enzymatic amplification in the microfluidic channel

A combination of electrochemical and enzymatic amplification processes was carried out for the detection of greater electrical signals. The electrochemical amplification can be obtained by redox cycling, which is related to the fast regeneration of the reduced ferrocene [Fc^{2+}] after the enzymatic oxidation of glucose.[43] Fig. 7 and 8 show the steps of catalytic electron transfer and anodic current arising procedures when GOx was mixed with a redox mediator, Fc-Lys-HA in the microfluidic channel. The difference in electrocatalytic anodic currents (i_{max}) from the control solution (concentration of HA: 0 mg mL^{-1}) and from the sample solution (concentration of HA: 40 mg mL^{-1}) of the enzymatic process was 3.1 times higher than that of the non-enzymatic process. The inset shows the calibration curves of the gold electrode at different concentrations of HA. As shown in the inset of Fig. 6, the detection of anodic currents is almost linear in the HA range of 0–30 mg mL^{-1}.

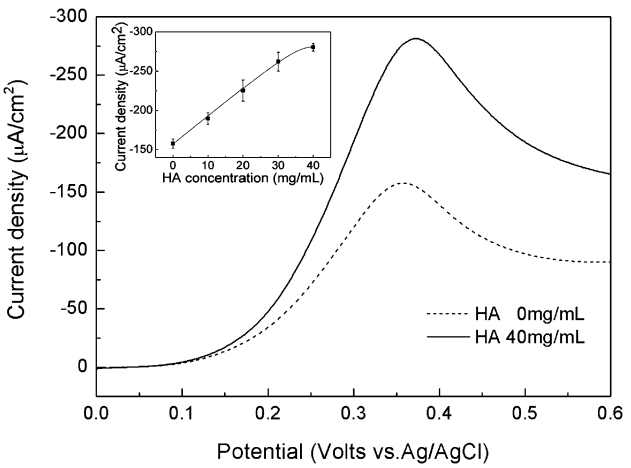

Fig. 8 Linear sweep voltammograms of the electrodes in 0.1 M PBS buffer (pH 7.2) made by 1.0 mg mL^{-1} Fc-Lys-HA with (solid line) and without (dash line) HA containing 1.0 mg mL^{-1} of GOx and 5 mM of glucose at scan rate of 5 mV s^{-1}. Inset: catalytic anodic currents as a function of the concentration of HA between 0 and 30 mg mL^{-1}. $R = 0.992$ (N = 4).

Real "spiked" HA sample analysis

In order to prove the practical applicability of this assay, we spiked HA into the human urine sample. Fig. 9 shows the cyclic voltammograms of the microfluidic immunoassay with Fc-Lys-HA, in the presence and absence of HA. In the pure PBS buffer system, the electrodes showed a quasi-reversible redox response of the ferrocene moieties in Fig. 6. In contrast, the human urine sample resulted in a dramatic increase in the anodic peak and a decrease in the reductive current. These facts 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.[44–46] Like the PBS solution, a similar amperometric peak was also observed in the human urine sample at 0.40 V (*vs.* Ag/AgCl). In addition, Fig. 9 shows the relation of the anodic current to the HA concentration, an increase of the HA concentration induces a higher current due to the higher concentration of Fc-Lys-HA. The inset of Fig. 9 illustrates the peak current magnitude ($i_{p,a}$) at 0.40 V (*vs.* Ag/AgCl) according to the concentration of HA (0, 10, 20, 30 mg mL^{-1}). The peak current increased linearly with the HA concentration with a correlation coefficient of 0.986, which shows that the microfluidic-based chip can detect the HA in human urine.

Discussion

In this paper, we tried to detect the HA as simply as possible and several studied were successfully carried out to this end. Firstly, we synthesized a single electroactive metal complex with one small antigen bound to a single metal atom (Fc-Lys-HA). According to the American Conference of Governmental Industrial Hygienists (ACGIH), the biological exposure index

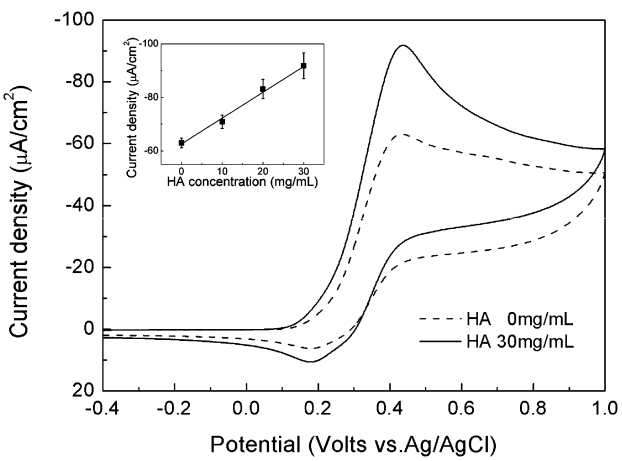

Fig. 9 Cyclic voltammograms of 1.0 mg mL^{-1} Fc-Lys-HA with (solid line) and without (dash line) HA in human urine samples at scan rate of 100 mV s^{-1}. Inset: peak currents as a function of the concentration of HA between 0 and 30 mg mL^{-1}. $R = 0.986$ (N = 3).

(BEI) of HA to define a significant toluene exposure is 2.0 mg of HA/mL in urine. This high concentration of HA generates unwanted noise when it is measured by the conventional method. However, when we use the Fc-Lys-HA complex, good calibration curves proportional to the concentration of HA can be obtained because one Fc-Lys-HA complex transfers one electron to the electrode by a simple redox reaction. In this case, confirming the synthesis of Fc-Lys-HA is important and we verified the conjugation and reduction reaction by thin layer chromatography (TLC) and ^{1}H NMR spectroscopy.

Secondly, the complicated electrochemical HA immunoassay process was successfully realized on the single microfluidic platform. The chip-based assay is very simple and all the process was carried out without the intervention of an operator, which reduces labor and cost. The quantitative advantages of HA detection based on our immunoassay chip are as follows: (1) the total chip size was reduced to $3.0 \times 2.0 \times 0.5$ cm and is small enough to be portable, (2) the assay time took 1 min, and is shorter than that of conventional electrochemical HA immunoassay systems (about 20 min) and (3) the 40 μL of the sample solution (a mixture of fixed 1.0 mg mL^{-1} of Fc-Lys-HA and HA with variable concentration) was enough to detect HA in the range of 0–40 mg mL^{-1}. Inferring from this quantitative evaluation, the proposed chip-based HA detection system can facilitate the point-of-care system for the ubiquitous monitoring of toluene exposure.

Finally, we suggest the improved chip-based HA assay method by the combination of electrochemical and enzymatic amplification processes for the detection of greater electrical signals. The current density of the combined method increased about three times more than that of the non-enzymatic process. This result indicates that the enzymatic process is more sensitive than the non-enzymatic one and is suitable for the detection of HA in microfluidic chips because electrochemical sensing is possible with a small amount of sample volume.

Conclusions

As conclusion, a microfluidic chip-based electrochemical HA immunoassay system was successfully developed, evaluated and showed feasibility for a portable toluene monitoring or detection system. To this end, several important studies, such as conjugation of HA and ferrocene (Fc), integration of several assay steps (reaction, separation by centrifuge and detection) into the single microfluidic platform and signal amplification by adding an enzymatic amplification process, were successfully carried out. With further improvement, the suggested system can be broadly used to the point of care system for the ubiquitous monitoring (or detection) of toxic small molecules or for the diagnosis of diseases.

Acknowledgements

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