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The Coordination of Pyridyl-N to Pentacyanoferrate for the Electrochemical Detecting Small Organic Molecules

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The coordination of pyridyl-N to pentacyanoferrate for the detection of small organic antigens in solution is presented. The unique contribution of this paper is the direct conjugation of pyridyl-N in small organic antigens to pentacyanoferrate. Pentacyanoferrate is promising as an electrochemical label owing to its good electrochemical properties, which can be utilized to generate an electrical signal in homogeneous electrochemical immunoassays. The facilely synthesized pyridyl-N to pentacyanoferrate was characterized by the electrochemical and spectroscopic methods. Hippuric acid (HA) has been detected competitively on the interaction of free HA and pentacyanoferrate-(4-aminomethylpyridine-hippuric acid) (Fe-HA) to its antibody, with the detection limit of 0.50 μg mL^{-1}. While pentacyanoferrate-based immunoassay is in its simplicity and infancy, the proposed immunoassay offers attractive opportunities for developing pyridyl-N-based the electrochemical detection of small organic antigens in the health care area.

Key Words : Antigen, Antibody, Homogeneous electrochemical immunoassay, Pyridyl-N, Pentacyanoferrate

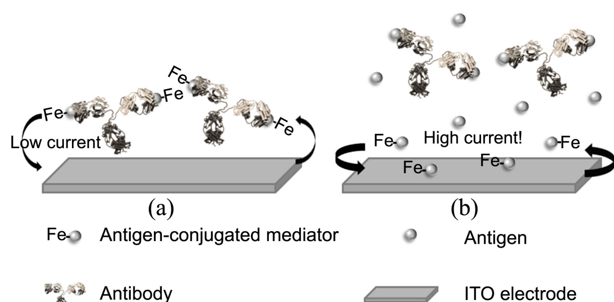
Introduction

The fast, simple, inexpensive and accurate method for the detection of small organic molecules (< 1000 Da), such as drugs, narcotics, pesticide residues and metabolites, is very important in the fields of public health, safety, and the environment. Immunoassays have been studied as a promising means of detecting small organic molecules, due to their ability to bind small target molecules with high specificity and sensitivity. Many kinds of immunoassay based on optical techniques have been applied for the detection of small organic molecules. Despite the many advantages in these methods, there is still room to improve their simplicity and portability and reduce their cost. In contrast, electrochemical immunoassays are attractive systems for the development of simple portable devices for on-site monitoring.[1-4]

In electroanalysis, metal hexacyanoferrate (MHCF) complexes such as NdHCF, SnHCF, CoHCF, and NiHCF, have been studied as redox mediators for chemical sensors and biosensors.[5-8] In addition, amperometric immunoassays based on nano-Au and NiHCF particles has been developed for the determination of carcinoembryonic antigens.[9] So far, little attention has been paid to the utilization of MHCF complexes to construct homogeneous immunoassays. Moreover, most of the homogeneous immunoassays that have been developed were based on the modulation of the enzymatic activity, followed by an immunological reaction. However, the preparation of enzyme-functionalized antigens/antibodies is relatively complex and shows low reproducibility.

Recently, we have developed a homogeneous electrochemical immunoassay based on ferrocene and osmium complexes with fast diffusion for the detection of HA.[10,11] In this paper, a facile synthetic process enabling the direct coordination of pyridyl-N to pentacyanoferrate for the detection of small organic molecules is reported.[12,13] In particular, two novel strategies are employed to design the homogeneous electrochemical immunoassay system, as follows: First, the synthesized complex between pyridyl-N and pentacyanoferrate was not only an excellent electroactive molecule, but also provided direct conjugation to the antibody for the detection of the target molecules. Second, the proposed homogeneous electrochemical immunoassay is relatively simple, inexpensive and easily manipulated.

Compared to heterogeneous immunoassays, homogeneous assays are relatively more simple and inexpensive.[14-17] However, the sensitivity of homogeneous assays is generally lower than that of heterogeneous ones.[18,19] Thus, we chose a small organic molecule, such as HA, with a high physiological concentration, which is a major urinary metabolite in toluene-exposed humans with a cutoff concentration of 2.0 mg mL^{-1} in urinary samples.[20,21] The principle of analysis is based on the decrease in the electrochemical signal of the HA antigen-conjugated Fe (Fe-HA) when it binds to its antibody (Scheme 1). Combining the Fe-HA with the antibody, which is relatively large, leads to a slow diffusion rate and obstructs the electron transfer of the Fe-HA, as compared to relatively small Fe-HA. In addition, the small Fe-HA is more competitive than the conjugated-enzymes antigens previously reported. The amperometric signal from the Fe-HA is directly correlated with the free HA concentration in the sample solution. The detection limit of this immunoassay system was one microgram mL^{-1} (> 1.0 μg mL^{-1}). Moreover, the



Scheme 1. Schematic illustrating the homogeneous electrochemical immunoassay. (a) In the absence of antigens, antibody is combined with the Fe-HA expected low current. (b) In the presence of antigens, antibody is competitively combined with antigens (HA and Fe-HA) expected high current.

proposed homogeneous electrochemical immunoassay method can be extended to the detection of a wide range of small antigens in the field of health care.

Experimental

Reagents. Monoclonal *anti*-HA (HA antibody) was kindly donated by HBI (Seoul, Korea). 4-Aminomethylpyridine, HA, buffering salts, and other chemicals were purchased from Sigma-Aldrich Co. (Milwaukee, WI, USA). Ammonium disodium pentacyanoamminferrate dehydrate was purchased from Fluka. Phosphate-buffered saline (PBS, 4.3 mM NaH_2PO_4, 15.1 mM Na_2HPO_4, and 140 mM NaCl) and all other solutions were prepared using deionized Milli-Q water (Millipore, Japan). All chemicals used were of analytical grade.

Preparation of $[Fe(CN)_5(amp\text{-}HA)]^{3-/2-}$ (Fe-HA). Aminomethypyridine-hippuric acid (amp-HA) was prepared by refluxing HA and 4-aminomethyl pyridine in ethanol and then precipitated using ethyl ether to produce a white powder (Fig. 1). The synthesis of amp-HA was confirmed by thin-layer chromatography (TLC) and ^1H NMR spectroscopy. The ^1H NMR spectrum (400 MHz, DMSO) was as follows: δ_{ppm} 7.45-7.95 (m, 5H, phenyl), 7.32-8.65 (m, 4H, Py), 3.98-4.25 (t, 4H, J = 7.0 Hz, $-CH_2-CH_2-$), 7.8-8.01 (m, 2H, -NH-NH-). As shown in Figure 1, 50.0 mg of $Na_2[Fe(CN)_5NH_3]\cdot 2H_2O$ and 44.4 mg of amp-HA were dissolved in 60 mL of aqueous ethanol solution (*v/v* : 50/50) and kept at room temperature for 24 h. After filtering, the solution was added to 2.0 L of diethyl ether with vigorously stirring. The product was precipitated, washed with ethanol and then dried for several hours in a vacuum oven. The conjugation of Fe-HA was verified by UV-vis and FT-IR spectroscopy (Figs. 2 and 3). The validation of Fe-HA as an antigen was performed using an immunochromatographic stripe from HBI (Seoul, Korea). 4 x PBS samples spiked with various concentrations of Fe-HA were assayed by an immunochromatographic detection device. The analysis was completed within 2 minutes.

Electrochemical Measurements. Electrochemical measurements were carried out with a CH Instruments model 660A electrochemical workstation (CH Instrument, Austin, TX, USA), interfaced to a computer. The electrochemical characteristics of Fe-HA were studied using 3.0 mm-diameter ITO electrodes as the working electrodes. An Ag/AgCl micro-reference electrode (3.0 M KCl, Cypress, Lawrence, KS, USA) scrolled with a 0.5 mm diameter platinum wire counter-electrode was used. Cyclic voltammetry (CV) and amperometric analyses were conducted with 0.4 mg mL^{-1} of Fe-HA dissolved in 0.1 M PBS buffer (pH 7.2).

The general process using the homogeneous electrochemical method is described in Scheme 1(a). Fe-HA and antibody can be mixed well and then loaded onto the electrode. In the absence of antigen, complexation of antibody and Fe-HA leads to the attenuated passage of current derived from the slow diffusion of Fe (II/III) redox probe in aqueous solution. The competitive electrochemical immunoassay method (Scheme 1(b)) is as follows: (1) Fe-HA and free antigen are competitively reacted with its antibody. (2) After antigen-antibody reaction, the amperometric signal from the electrochemically active Fe-HA can be finally measured. The amperometric signal increased upon the interaction of antibody and free antigen; an event motivated by the presence of free antigen in the solution. The amperometric signals from Fe-HA are directly correlated with free antigen concentrations in sample. The detail feasibility of HA immunoassay was performed as the following steps: (i) 40 μL of 0.15 mg mL^{-1} of Fe-HA with various concentrations of *anti*-HA samples was loaded onto ITO electrodes, and incubated for 20 minutes at room temperature with PBS, pH

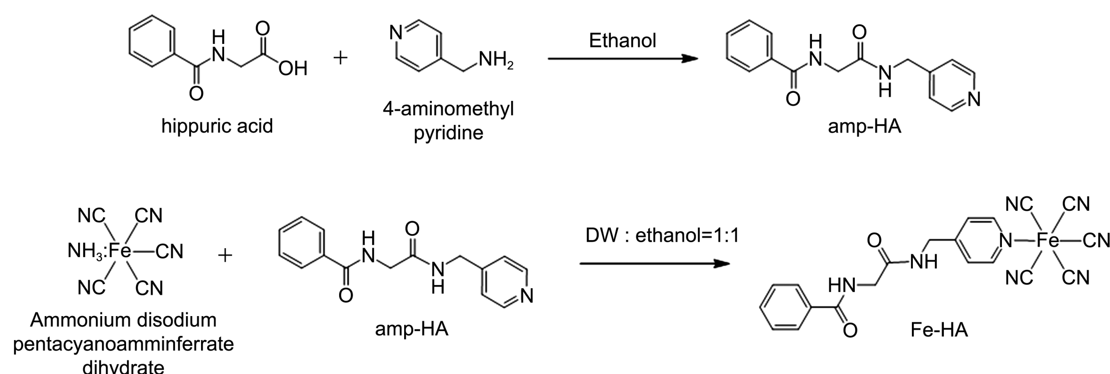


Figure 1. Preparation of Iron-conjugated HA antigen $[Fe(CN)_5(amp\text{-}HA)]^{3-/2-}$ (Fe-HA).

7.2; (ii) the results of DPV from - 0.2 to 0.6 V *vs* Ag/AgCl with a pulse amplitude of 0.05 V and a pulse width of 50 ms was collected and plotted as the electrochemical immunosensors signal. The competitive immunoassay of HA is also carried out as followed: (i) 40 μL of 0.15 mg mL^{-1} of Fe-HA and 0.5 mg mL^{-1} of *anti*-HA with various concentrations of HA solution was loaded onto the ITO electrodes, and incubated for 20 minutes at room temperature with pH 7.2 PBS; (ii) after the competition reaction, the unbounded Fe-HA was electrochemically measured by DPV from - 0.2 to 0.6 V *vs* Ag/AgCl with a pulse amplitude of 0.05 V and a pulse width of 50 ms. All the baselines of CV or differential pulse voltammetry (DPV) were corrected by CHI660A software.

Results and Discussion

Spectroscopic Characteristics of $[Fe(CN)_5(amp\text{-}HA)]^{3-/2-}$ (Fe-HA). The direct conjugation of pyridyl-N in small organic antigens to pentacyanoferrate was studied with UV-visible spectroscopy as shown in Figure 2, in which the UV-visible spectra of $Fe(CN)_5NH_3$ (a) and Fe-HA (b) dissolved in water are displayed. The UV-visible spectrum of $Fe(CN)_5NH_3$ at a concentration 0.33 mM shows a strong absorption at 400 nm and a broad absorption band at 360 nm [$\varepsilon = 6.25 \times 10^2$ and 6.04×10^2 M^{-1} cm^{-1}, respectively]. With the first equivalent (a) transforms into (b) almost quantitatively, as evidenced by the characteristically strong absorbance peak at 380 nm [$\varepsilon = 1.86 \times 10^3$ M^{-1} cm^{-1}], which was increased about three times compared to that of $Fe(CN)_5NH_3$. The formation of Fe-HA was also investigated by FT-IR spectroscopy, as shown in Figure 3. The FT-IR spectrum of $Fe(CN)_5NH_3$ exhibits two N-H stretching bands in the range of 3500-3300 cm^{-1} (Fig. 3(a)), whereas Fe-HA show only one band in that range (Fig. 3(b)).

Electrochemical characteristics of $[Fe(CN)_5(amp\text{-}HA)]^{3-/2-}$ (Fe-HA). From the cyclic voltammetry of Fe-HA in 0.1 M PBS buffer (pH 7.2) on the ITO electrodes, Fe-HA showed one pair of *quasi*-reversible redox peaks at $E_{1/2} = 0.22$ V *vs* Ag/AgCl, as shown in Figure 4. These results suggested that Fe-HA was a fast and reversible redox mediator that could be suitable for the homogeneous electrochemical immunoassay of HA. The steady-state cyclic voltammograms with

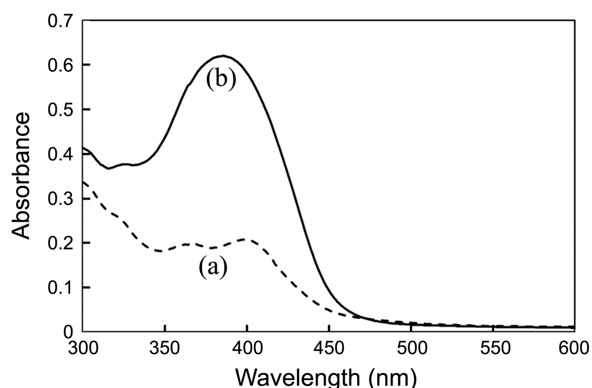


Figure 2. UV-Visible spectra (a) $Fe(CN)_5NH_3$ and (b) Fe-HA.

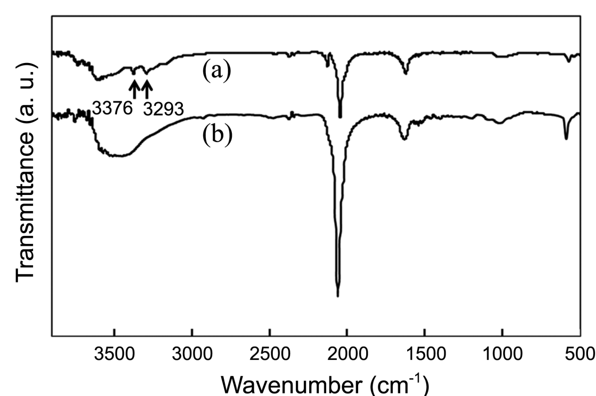


Figure 3. FT-IR spectra (a) $Fe(CN)_5NH_3$ and (b) Fe-HA.

Fe-HA on ITO electrodes are also shown in the inset of Figure 4 as a function of the scan rate. The inset in Figure 4 shows that the anodic peaks [$(I_p)_a$] and the cathodic peaks [$(I_p)_c$] of Fe-HA increased linearly with the square root of the scan rate ($\nu^{1/2}$) in the range of 0.01-0.1 V s^{-1}. This result suggests that the electron transfer process on the electrode is controlled by the diffusion reaction.

Optimization of Homogeneous Immunoassay Conditions. During the electrochemical measurements, the performance of the immunoassay was affected by the pH, incubation temperature and incubation time. To achieve a steady electrical signal, the temperature, pH and incubation time should be controlled. All experiments were carried out at room temperature (25 ± 1 °C) and under physiological conditions of 0.1 M PBS (pH 7.2) with 0.14 M NaCl in order for the system to be applicable to practical applications. At room temperature, the effect of the incubation time on the current of the $Fe^{2+/3+}$ moieties was monitored at 5, 10, 15, 20, 25, 30, 35, and 40 minutes with 0.5 mg mL^{-1} of *anti*-HA and 0.15 mg mL^{-1} of Fe-HA in PBS (pH 7.2). The electrochemical response decreased with increasing incubation time and reached a plateau after 20 minutes. A longer incubation time

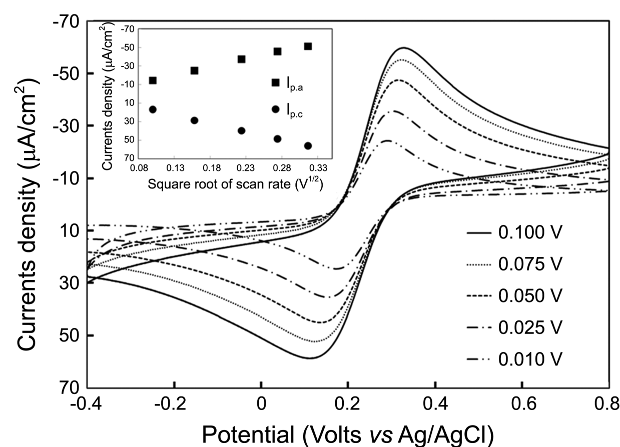


Figure 4. Cyclic voltammograms of 0.4 mg mL^{-1} of Fe-HA dissolved in 0.1 M PBS buffer (pH 7.2) with 0.14 M NaCl on the ITO electrode at different scan rates (0.01, 0.025, 0.05, 0.075, 0.10 V s^{-1}). Inset: The variation of the peak currents density *vs* the square root of the scan rate. $R_1 = 0.9985$ ($I_{p,a}$, square), $R_2 = 0.9995$ ($I_{p,c}$, dot).

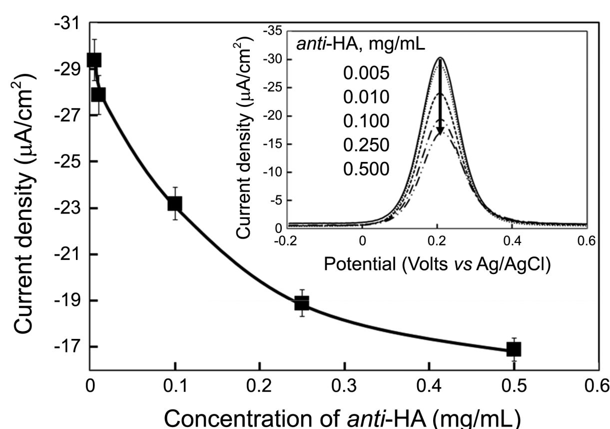


Figure 5. Calibration curve of the anodic DPV peak current of 0.15 mg mL^{-1} of Fe-HA at 0.22 V *vs* Ag/AgCl as a function of the *anti*-HA concentration between 0.005 and 0.50 mg mL^{-1}. Reaction conditions: in PBS (pH 7.2, 0.14 M NaCl) with a pulse amplitude of 0.05 V and pulse width of 50 ms in DPV. Inset: DPV curves.

(> 20 minutes) did not change the electrical signal. Therefore, an incubation time of 20 minutes was adopted throughout the detection of HA.

Electrochemical Responses of the Immunosensor with Increasing *anti*-HA Concentrations. The calibration curves for the HA immunosensor with increasing concentration of *anti*-HA are presented in Figure 5. The role of Fe-HA is as a redox mediator which transfers electrons to the electrodes and the small organic antigen which can be conjugated with the *anti*-HA in the immune reaction. The inset in Figure 5 shows the typical DPV of the Fe-HA based on the different *anti*-HA concentrations. Low electrical signals were observed at high *anti*-HA concentrations, whereas high electrical peaks of the Fe-HA were observed at low *anti*-HA concentrations. From these results, it was found that Fe-HA firmly binds to the *anti*-HA and produces a complex of Fe-HA with the *anti*-HA, which is much heavier than the Fe-HA itself in terms of its molecular weight and size. In the present work, the molecular weight of the *anti*-HA is approximately 150 kD, which is 100 times heavier than the Fe-HA. As shown in Figure 5, the electrical response of Fe-HA to increasing concentrations of the *anti*-HA was analyzed by measuring the current magnitude ($i_{p,a}$) of $Fe^{2+/3+}$ at 0.22 V *vs* Ag/AgCl. The decrease in the Fe-HA current signal upon the binding of the *anti*-HA was attributed to its inhibiting Fe-HA from moving to the electrode, which hinders the electron transfer between the $Fe^{2+/3+}$ moieties. Also, the anodic current decreased non-linearly, which implies that the kinetics approached saturation at approximately 0.5 mg mL^{-1} of *anti*-HA. The attenuation of the anodic current was proportional to the concentration of the *anti*-HA in the detection range of 0.005-0.50 mg mL^{-1} with a low detection limit (LOD) of 1.0 μg mL^{-1} of *anti*-HA with a signal to noise ratio of 3σ.

Competitive Immunoassay for HA Analysis. Under the optimal conditions, the competitive immunoassay with Fe-HA showed typical DPV curves in the presence and absence of HA, as shown in the inset of Figure 6. In accordance with

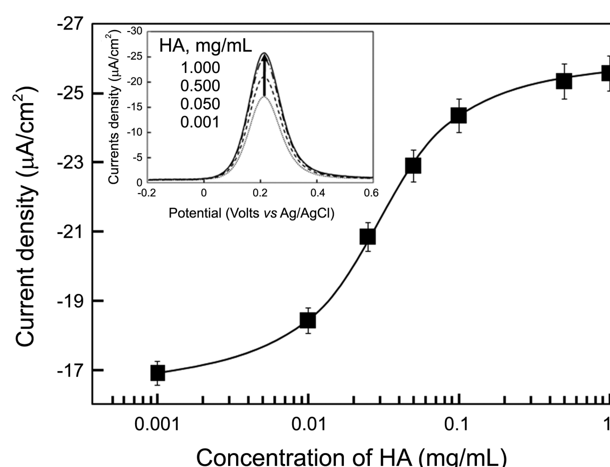


Figure 6. Calibration curve of the anodic DPV peak current of 0.15 mg mL^{-1} of Fe-HA *vs* logarithm of HA concentrations ranging from 0.001 mg mL^{-1} to 1.00 mg mL^{-1} (N = 4). Reaction conditions: fixed *anti*-HA concentration of 0.5 mg mL^{-1}, in PBS (pH 7.2, 0.14 M NaCl with a pulse amplitude of 0.05 V and pulse width of 50 ms in DPV. Inset: DPV curves.

the competitive reaction between HA and Fe-HA antigen with the *anti*-HA, the anodic current from Fe-HA was directly correlated with the HA concentration. In the presence of HA, high DPV peaks of Fe-HA result from the competition reaction between HA and Fe-HA for the *anti*-HA. In addition, the small Fe-HA in this work is more competitive than the antigen with the conjugated enzymes previously reported. As shown in Figure 6, the magnitude of the anodic current ($i_{p,a}$) at 0.22 V *vs* Ag/AgCl was chosen to represent the concentration of HA. Some of the interfering compounds such as ascorbic acid, acetaminophen and uric acid in physiological samples could be negligibly oxidized at 0.2 V *vs* Ag/AgCl. The increase of the anodic peak current was proportional to the logarithm of the HA concentration in the detection range of 0.001-1.0 mg mL^{-1} with an LOD of 0.5 μg mL^{-1} (N = 4, r = 0.965). N denotes the number of different electrodes used. Therefore, this new homogeneous electrochemical immunoassay is suitable for the detection of HA in physiological samples.

Conclusions

We demonstrated for the first time the use of the coordination of pyridyl-N to pentacyanoferrate for the electrochemical detection of small organic antigens. A proposed homogeneous electrochemical immunosensor was designed using the direct binding of a single pentacyanoferrate to a small organic antigen, HA. The direct binding of pyridyl-N containing HA to pentacyanoferrate was verified by UV-visible, FT-IR and electrochemical measurements. In conclusion, the newly synthesized Fe-HA shows good electrochemical properties which allow it to transfer electrons to electrodes and behave as a small organic antigen which can be conjugated with *anti*-HA in homogeneous electrochemical immunoassays. With further improvement, the proposed immunoassay system could provide a new method for the

highly selective, sensitive and reproducible label-free detection of different small organic antigens in the field of point-of-care diagnosis.

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