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A New Electrochemical HbA_{1c} Biosensor Based on Flow Injection and Screen-Printed Electrode

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In this paper a novel elctrochemical HbA_{1c} biosensor based on flow injection and screen-printed electrode was developed. The reticulated vitreous carbon (RVC) electrode was modified with 3-aminophenylboronic acid, chitosan (CS), and tetraenthoxyl silica (TEOS), which entrapped carbon nanotubes to form a sol-gel film at the surface of the screen-printed electrode. The electrochemical behavior of the biosensor was investigated using cyclic voltammograms and amperometric method. Then the reusability of RVC electrode was investigated at various pH values. Furthermore, the selectivity and stability of the HbA_{1c} biosensor was studied. The biosensor was also employed to detect the real samples, and the results were compared to automatic biochemical analysis.

Keywords: HbA_{1c}; screen printed electrode; electrochemical biosensor; reusability

1. INTRODUCTION

Hemoglobin A_{1c} (HbA_{1c}), a specific glycated hemoglobin, is formed irreversibly through a nonenzymatic reaction of glucose and the N-terminal valine of the β-chain in hemoglobin[1]. The level of HbA_{1c} reflects the average blood glucose concentration over the preceding 2 to 3 months. So it is the most significant index for diabetes or related diseases. The normal level of HbA_{1c} is lower than 6%, whereas diabetes can be diagnosed when the level of HbA_{1c} is higher than 6.5%[2]. Thus it is important to control the level of HbA_{1c}, which is a diagnostic criterion for diabetes.

There are several methods to detect the ratio of HbA_{1c} to total hemoglobin (%HbA_{1c}), such as surface plasmon resonance (SPR), chemiluminescence flow cells, electrophoresis, ion-exchange high performance liquid chromatography (HPLC)[3], fluorescent sensor, and boronate affinity

chromatography. Most of those methods have common disadvantages including long time of analysis and high cost for each test[4-6]. So it is the trend to search for a method with simplicity, low-cost, and sensitivity. The diol group of boronic acid can bind with the cis-diol group of the sugar from HbA_{1c} under alkaline conditions[1,2,7]; thus it is used to capture glycated protein and the HbA_{1c} can be separated from hemoglobin. Whereas Hb contains four iron heme groups, Hb or HbA_{1c} can be oxidized at a special electrode. Baccarin *et al.* developed a poly(amidoamine)-dendrimer-modified glassy carbon electrode to realize direct electron transfer from Hb[8].

The electrochemical biosensor, especially disposable electrochemical sensors based on screen-printed electrode (SPE) technology, has the advantages of low-cost, simplicity, high sensitivity, and high efficiency[9-14]. It has been applied in various fields such as clinically, environmentally and in food production. The aim of this work is introducing of a rapid, simple and sensitive HbA_{1c} biosensor based on flow injection and screen-printed electrode. Reticulated vitreous carbon (RVC), with low electrical resistance and high current density, is used as the porous electrode[15-17]. The RVC electrode is modified with 3-aminophenylboronic acid. When the sample is injected, HbA_{1c} can bind with the RVC electrode, and Hb can be separated and oxidized at the SPE. On account of the biocompatibility and electrocatalyzing potential of carbon nanotubes (CNT)[18], the material was employed and entrapped to the SPE by chitosan (CS) and tetraenthoxyl silica (TEOS), forming a sol-gel film. Judged by investigation of the electrochemical behaviors of the HbA_{1c} biosensor, the method can have clinical application.

2. EXPERIMENTAL

2.1 Materials

The reticulated vitreous carbon (RVC) foam was purchased from Aerospace (Oakland, CA, USA). Ascorbic acid, uric acid, acetaminophen, 3-aminophenylboronic acid, chitosan(CS), tetraenthoxyl silica(TEOS), hemoglobin(Hb), N-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HbA_{1c} calibrators were obtained from Primus (Kansas, MO, USA).

2.2 Preparation of boronate-modified RVC electrode

The reticulated vitreous carbon (RVC) electrode was prepared as follows: the RVC foam was compressed 10 times and designed as the working electrode with a diameter of 3 mm and a length of 5 mm, and then the RVC electrode was immersed in the mixed solution of HNO_3 and H_2SO_4 with a volume ratio of 3:1 to react 1 h at 85 °C. Then the RVC electrode was washed and immersed 1 h in the mixed PBS solution having a pH of 5.5 and containing 5 mmol/L EDC and 5 mmol/L NHS. After washing with water the RVC electrode was reacted with 10 mmol/L 3-aminophenylboronic acid for 1h, and then 5 mmol/L glycine was employed to block the unreacted carboxyl of RVC. Then the boronate-

modified RVC electrode was set into homemade cylindrical PEEK to form the flow cell. The inlet of the RVC electrode assembly was connected to a six-port rotary valve.

2.3 Preparation of Hb-detecting electrode based on the screen-printed electrode

The hemoglobin (Hb) detecting electrode was prepared by using chitosan(CS) and tetraenthoxyl silica(TEOS) to entrap carbon nanotubes, forming a sol-gel film on the screen-printed electrode. The ratio of CS with TEOS was 1:1. The screen-printed electrode was dipped into the mixed solution (10 μL) and dried at 45 °C for 10 min. Then the direct electrochemical behavior of Hb was investigated using cyclic voltammetry. The potential was in the range from -0.65 V to 0.15 V, and the scan rate was 100 mV/s. The chronoamperometric detection of Hb was done under a potential of -0.2 V. The modified screen-printed electrode was mounted to the thin-layer cell body and connected with the RVC electrode with a nut.

2.4 Electrochemical flow injection assay system of HbA_{1c}

All the electrochemical measurements were carried out with a model CHI832C Electrochemical Workstation (CH Instruments, Austin, TX, USA). The flow rate was 1.0 mL/min. The potential at the screen-printed electrode was set at -0.2 V. The carrier solution was PBS solution with pH of 8.0. 10 μL of sample or whole blood was added into 10 μL red cell lysis buffer and reacted 30 s, and then the solution was injected through a valve by a syringe pump. After the signal at the screen-printed electrode decreased, a potential of 0.2 V was set at the RVC electrode. Based on calculation of the ratio of electric charge at the RVC electrode and the screen-printed electrode, the $\%HbA_{1c}$ can be obtained. After amperometric measurements in PBS solution, the RVC electrode can be reused after 1-min replacement of the carrier solution with acetate buffer with a pH of 4.5. Then the RVC electrodes were kept dry at room temperature. All experiments were carried out at room temperature unless otherwise stated.

2.5 Real sample preparation and analysis

Whole blood samples (2.0 mL each) were collected into vacuum tubes containing EDTA. Then the samples were added to 2 mL red cell lysis buffer. $\%HbA_{1c}$ of the blood samples can be obtained by calculating the ratio of the electric charges at the RVC electrode and the screen-printed electrode. The results were compared to those determined with an standard HbA_{1c} analyzer which used the HPLC method (Bio-Rad VARIANT™ II Hemoglobin Testing System, USA).

2.6 Reference method for HbA_{1c} determination

HPLC is the reference method for HbA_{1c} determination developed by the International Federation of Clinical Chemistry and Laboratory Medicine[2]. It is used by many laboratories in the

United States. The method requires anticoagulated blood which is diluted with a hemolysis reagent. Apart from the hemolysis process, samples usually need pretreatment by incubation at 37 °C for 30 minutes, whereby an interfering substance is removed. Then a series of three phosphate buffers (of increasing ionic strength) was passed through the column. Light absorbance is read at two different wavelengths, a curve is obtained, and integration is performed to calculate the area under the peaks[2].

3. RESULTS AND DISCUSSION

3.1 The electrochemical behavior of Hb at screen-printed electrode

Cyclic voltammetry (CV) was employed to investigate the direct electrochemical behavior of hemoglobin (Hb). Chitosan (CS) and tetraenthoxyl silica (TEOS) was employed to entrap CNT to the screen-printed electrode. And the ratio of CS to TEOS was also investigated. When the ratio was 1.0, Hb can be oxidized directly at the screen-printed electrode. The oxidation potential and the reduction potential were -0.21 V and -0.34 V respectively as shown in Figure 1. The results revealed that CNT can facilitate the electron transfer between Hb and the screen-printed electrode.

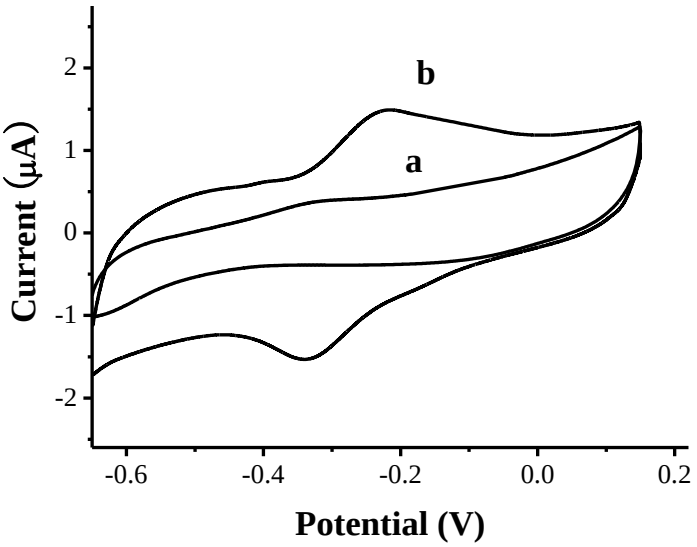

Figure 1. Cyclic voltammograms of PBS solution containing 0(a) or 100 mg/mL (b) Hb at the screen-printed electrode. The scan rate was 100 mV/s.

When the modified screen-printed electrode was mounted to the thin-layer cell body, the flow rate was investigated as shown in Figure 2. When the flow rate was lower than 1.0 mL/min, the electric charge at the screen-printed electrode increased with the flow rate. When the flow rate was higher than 1.2 mL/min, the electric charge decreased with the flow rate, which was attributed to the incomplete oxidation of Hb when the flow rate was too high. So we chose the flow rate of 1.0 mL/min.

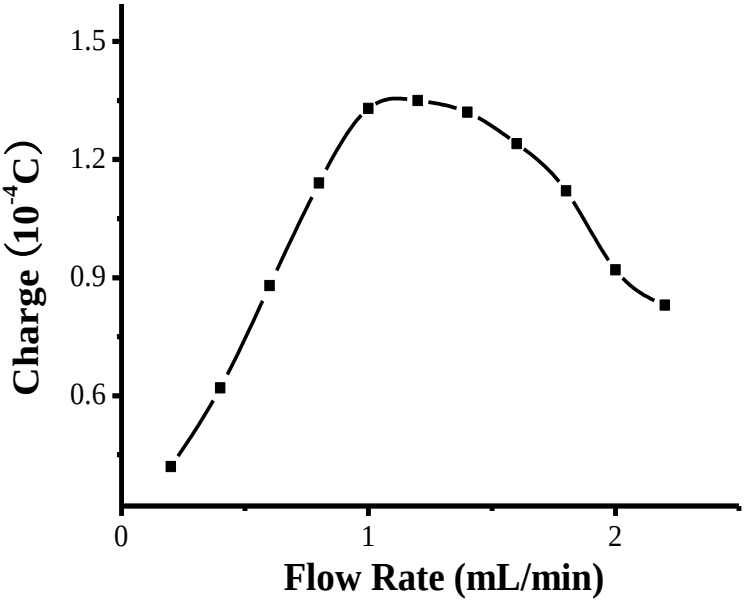

Figure 2. The relationship between flow rate with electric charge when Hb concentration was 100 mg/mL and the potential was -0.2 V.

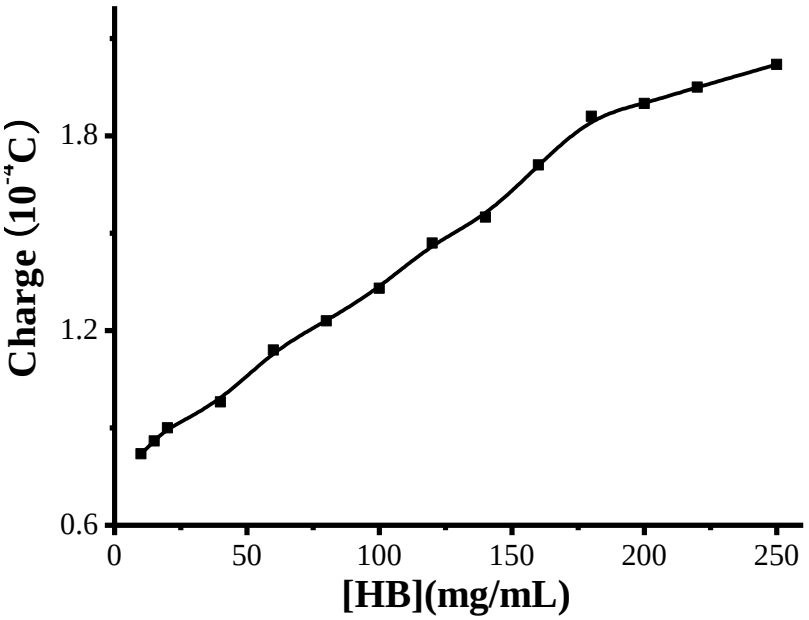

Figure 3. The relationship between Hb concentration (from 10-250 mg/mL) and electric charge of the chronoamperometry curve when the potential was -0.2V.

Chronoamperometry was used to detect the relationship between Hb concentration and electric charge when the potential was set at -0.2 V. The results were shown in Figure 3, the plot of the electrocatalytic electric charge at screen-printed electrodes in relation to Hb concentration in the range of 10 mg/mL to 250 mg/mL. There was a linear relationship when Hb concentration was between 20 mg/mL and 200 mg/mL, and the linear regression equation was Q(c) (10^{-4}C) =0.0058 c(Hb) (mg/mL) + 0.77. The limit of detection was calculated as three times the background noise, which was 16 mg/mL.

3.2 The electrochemical behavior of HbA_{1c} at RVC electrode

As known, the diol group in boronic acid can bind with the cis-diol group in sugar from HbA_{1c} under alkaline conditions[1,2,7], so 3-aminophenylboronic acid was employed to modify the reticulated vitreous carbon (RVC) electrode. When the sample was injected into the porous RVC electrode, HbA_{1c} can bind with the group in boronic acid at the modified RVC electrode, thus HbA_{1c} can be separated from the sample. When the potential was set at 0.2 V, HbA_{1c} can be oxidized at the RVC electrode because of the low electrical resistance and high current density of RVC. As shown in Figure 4, there was a good linear relationship between HbA_{1c} concentration and electric charge with HbA_{1c} concentrations between 0.2 mg/mL and 12.0 mg/mL, and the linear regression equation was Q(c) =0.027 c(HbA_{1c}) (mg/mL) + 0.55. The limit of detection as calculated by multiplying the background noise by 3 was 89 μg/mL. The final result is %HbA_{1c}, which is the ratio of charges at the RVC and the SPE.

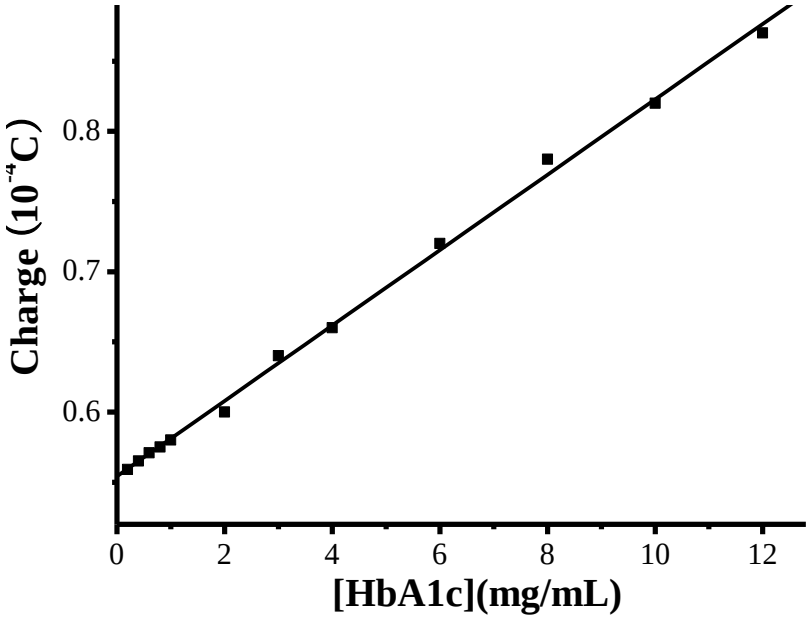

Figure 4. The calibration curve of HbA_{1c} concentration (from 0.2-12.0 mg/mL) with oxidization current of the chronoamperometry curve.

Our method attained a lower limit of detection than other tests of HbA_{1c}. For example, Chawla and Pundir developed a amperometric biosensor of HbA_{1c} based on immobilization of fructosyl amino acid oxidase onto a hybrid film. It had a detection limit of 50 μmol/L fructosyl valine (a proteolysis product of HbA_{1c})[5], which corresponded to 1.6 mg/mL HbA_{1c}. The better performance of our test is attributed to the use of RVC electrode, which has lower flow resistance and much larger specific surface area.

3.3 Effects of pH on RVC electrode reusability

To investigate the reusability of the RVC electrode, various pH was employed. Given the electric charge determined at the RVC electrode, we can calculate the HbA_{1c} remaining at RVC electrode. It is easier for HbA_{1c} to bind with boronic acid in alkaline conditions, so to reuse the RVC electrode, HbA_{1c} can be dissociated with pH lower than 5.0. Thus the best pH for RVC electrode reusability was 4.0-5.0. We chose pH of 4.5 in this research, and the washing time was 1 min.

3.4 Selectivity and stability of the HbA_{1c} biosensor

The electrochemical biosensor is often subject to interference by the oxidization or reduction of redox-active species such as uric acid (UA), ascorbic acid (AA), and acetaminophen (ACP) [19-21]. So it is necessary to study the selectivity and specificity of the HbA_{1c} biosensor. As shown in Figure 5, when the potential at the screen-printed electrode was -0.2 V, the electrochemically active species caused no interference in Hb detection because those species cannot be oxidized at such a low potential, so the results demonstrated that the HbA_{1c} biosensor had good selectivity and specificity.

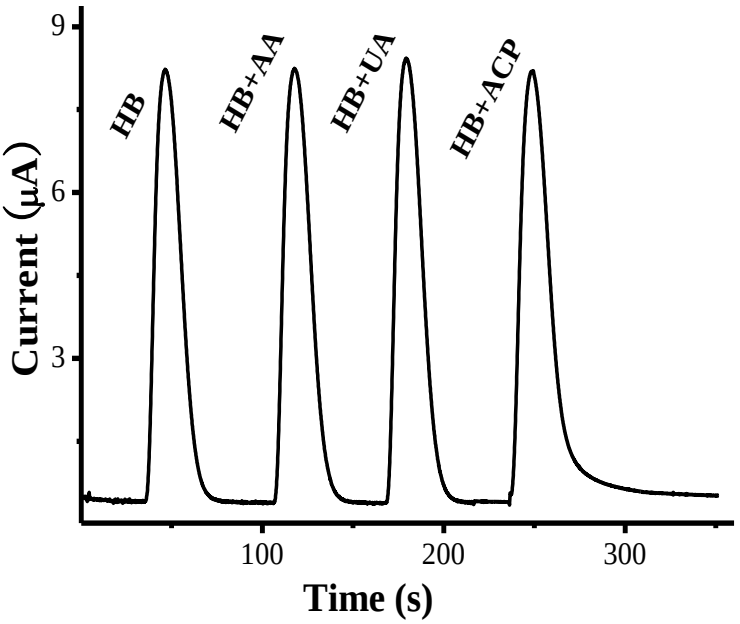

Figure 5. Selectivity of the HbA_{1c} biosensor for Hb in the absence or presence of redox-active species (AA, ACP and UA), the concentration being 100 mg/mL.

Some other studies also dealt with the interference issue in HbA1c measurements. For example, Chawla and Pundir's amperometric biosensor had a signal decrease of 8.85% with ascorbic acid and 21.28% with uric acid[5]. They considered those fractions negligible while we excluded the possibility of interference by using the low potential.

To investigate the stability of the HbA_{1c} biosensor, 2.5 mg/mL HbA_{1c} for the RVC electrode and 50 mg/mL Hb for the screen-printed electrodes (same bath) were employed. After each

measurement of HbA_{1c} at the RVC electrode, the electrode was washed with acetate buffer with the pH of 4.5 for 1 min. The RVC electrode lost 4.2% of its initial activity after 100 times used, which demonstrated that the RVC electrode had good stability. Due to the stability of the sol-gel film on the screen-printed electrodes[22], the same batch of electrodes had good repeatability for determination of Hb. Thus the HbA_{1c} biosensor can be used for real samples.

3.5 Real sample measurement

Whole blood samples (2.0 mL each) were collected into vacuum tubes containing EDTA. 10 μL sample was added into 10 μL red cell lysis buffer and reacted 30 s, the solution was injected through a valve by a syringe pump at a flow rate of 1.0 mL/min, then the potential at the screen-printed electrode was set at -0.2 V, and the chronoamperometry curve was recorded to detect Hb. When the signal kept constant, the potential at the RVC electrode was changed to 0.2 V to detect HbA_{1c}, and then we could calculate $\%HbA_{1c}$ based on the ratio of electric charge at the RVC electrode and the screen-printed electrode. The results were compared to those determined with an HPLC-based standard HbA_{1c} analyzer (Bio-Rad VARIANT™ II Hemoglobin Testing System, USA). As shown in Table 1, the results based on our method were in excellent agreement with the commercial automatic clinical analyzer. Compared with the clinical analyzer, there was no significant difference in accuracy. Our HbA_{1c} biosensor based on flow injection and screen-printed electrode had lower cost, was easier to prepare, and the results could be acquired in less than 5 min, which enabled clinical application.

Table 1. Comparison of $\%HbA_{1c}$ determined by our HbA_{1c} biosensor and HPLC, the reference method (example data)

Samples	HbA_{1c} biosensor	HPLC (Bio-Rad VARIANT™ II)
1	5.58%	5.62%
2	7.43%	7.55%
3	10.12.%	9.94%
4	8.67%	8.82%

4. CONCLUSIONS

In this paper we developed a novel electrochemical HbA_{1c} biosensor based on flow injection and screen-printed electrode. The RVC electrode was modified with 3-aminophenylboronic acid. Carbon nanotubes were entrapped by chitosan (CS) and tetraenthoxyl silica (TEOS) to form a sol-gel film at the surface of the screen-printed electrode. Hb could undergo direct electrochemical oxidation at the surface of the screen-printed electrode. There was good relationship between the concentrations of Hb (from 20 to 200 mg/mL) and electric charge of the chronoamperometry curve when the potential was -0.2 V at the screen-printed electrode. There was also good relationship between the HbA_{1c} concentrations (0.02~2.2 mg/mL) and electric charge when the potential was 0.2 V at RVC electrode.

The RVC electrode was good. Furthermore, the biosensor exhibited good reusability when pH was 4.5, and also selectivity and stability. This method was also successfully employed to test blood samples as compared with the automatic clinical analyzer. Those results revealed that the method could have clinical application.

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