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Electrochemical Analysis of Glycated Hemoglobin Based on the Biospecificity and Electron-Transferring Capability of Ferroceneboronic Acid

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Abstract

We have developed a biosensing strategy for glycated proteins in red blood cells. Glycated hemoglobin (HbA_{1C}) is recognized as an important target molecule to improve diabetes control and treatment. We employed ferroceneboronic acid (FcBA) as the signaling molecule, having both a signaling capability and biospecificity for target proteins. The boronic acid functional group in FcBA has an ability to form a covalent bond between its diol group and the *cis*-diol group from the carbohydrate chain on the surface of HbA_{1C}. The FcBA was reacted with samples containing HbA_{1C}, and the resulting conjugate was purified by a CENTRICON® membrane. After the solution-phase purification and concentration adjustment step, the electrochemical signal was registered. From the cyclic voltammetry, the detection range obtained for HbA_{1C} was around 0.1-1,000 μg/mL. By employing bioelectrocatalytic signal amplification on the glucose oxidase (GOX)-modified electrode, we were able to enhance the biosensor sensitivity. This study can be implemented in a diagnostic biosensor and is applicable to other glycoproteins having analytical needs.

Keywords: Glycated hemoglobin, HbA_{1C}, Ferroceneboronic acid, Electrochemical biosensor

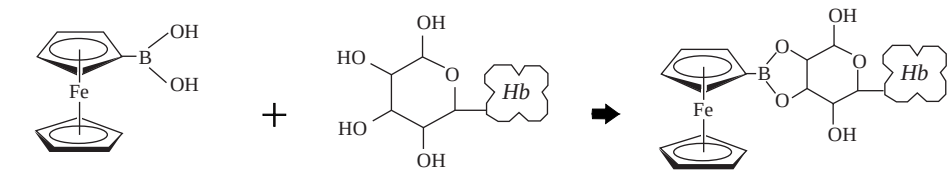
Introduction

We are witnessing a rapidly aging society and an increase in senior patients, leading to an intensified interest in the healthcare and expansion of the diagnosis field. In diagnostics, biosensor technology has drawn much interest. Biosensors are relatively small and handy devices using biochemical molecular recognition properties for a selective analysis[1]. Up until now, the most successful field of commercialization in biosensors has been glucose sensors for diabetes control. It has been certified that the annual growth rate of glucose sensors in the world market is around 13%. Diabetes mellitus is a disease caused by a high level of blood glucose resulting from insulin defection and is closely related to other serious complications and diseases[2]. Therefore, it is critical to develop an easy and efficient detection of glucose for both the diagnosis and treatment phases of diabetes.

As another marker for diabetes, glycated hemoglobin (HbA_{1C}) is in the spotlight as a next-generation blood glucose monitoring system. HbA_{1C} is made by a non-enzymatic reaction between glucose and the N-terminal valine of the β-chain of hemoglobin in a red blood cell. Because the lifespan of erythrocyte (approximately 100-120 days) is relatively long[3], the determination of HbA_{1C} helps in monitoring the long-term progression of diabetes without the influence of short-term fluctuations in blood glucose level. Therefore, HbA_{1C} concentration as well as blood glucose level should be selected as biosensing targets in order to improve diabetes control and treatment.

The detection principle of HbA_{1C} is based on its unique molecular feature. HbA_{1C} is a glycoprotein, having carbohydrate moiety on its three-dimensional molecular surface that can be employed as a targeting site. In order to capture and detect glycoproteins, we need an anchor molecule that can selectively bind to the target carbohydrates[4-6]. It has been reported that the boronic acid group has an affinity to carbohydrates, forming a covalent bonding between its diol group and *cis*-diols from them[7-13]. For the biosensing application of this biospecific reaction between boronic acid and glycorproteins (HbA_{1C}), we employed a compound containing boronic acid and ferrocenyl groups together, possessing an electrochemical activity and the ability to be involved in enzyme-catalyzed signaling[14]. To fulfill these conditions, ferroceneboronic acid (FcBA) was selected[15]. FcBA was conjugated to HbA_{1C} in solution under mild conditions, as shown schematically in Figure 1. The resulting HbA_{1C}-FcBA conjugate was separated and analyzed using electrochemical methods.

In addition, enzyme-mediated bioelectrocatalysis



Ferrocene boronic acid (FcBA) HbA_{1C} $FcBA\text{-}HbA_{1C}$

Figure 1. Biospecific covalent bonding between FcBA and HbA_{1C}.

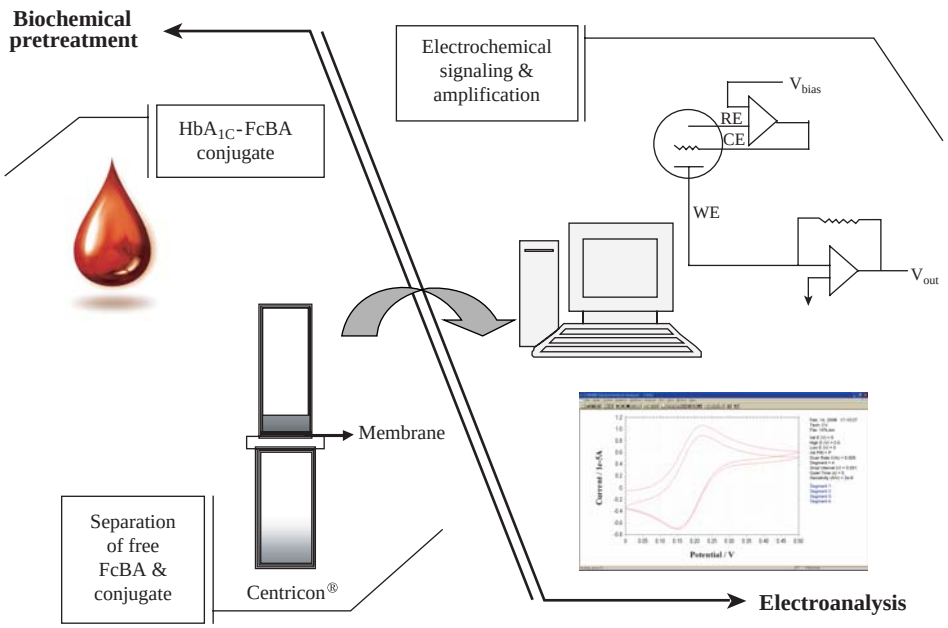


Figure 2. Schematic overview of the HbA_{1C} detection method, divided into a biochemical pretreatment and electroanalysis processes.

was employed to amplify the sensor signal due to the limited sensitivity from the direct electroanalysis of the HbA_{1C}-FcBA conjugate. Glucose oxidase (GOX) was employed as the signal generator in this case and was decorated on a self-assembled monolayer on gold electrodes[16-19]. Cyclic voltammetry was chosen for the signal registration. By using an anchor molecule for the target HbA_{1C} having biospecificity and electrochemical activity, and using an enzyme-mediated bioelectrocatalytic method, an efficient biosensing strategy for glycoproteins has been developed.

Results and Discussion

Measurement of HbA_{1C} through the Detection of Free FcBA

We have developed an electrochemical signaling method for HbA_{1C} in biological samples. The overall experiments conducted are summarized in Figure 2. The first part of the study is the induction of conjugation reaction between target molecule and FcBA, and then the separation of the conjugate from unreacted reagents. First, a target molecule (HbA_{1C}) was conjugated with FcBA using a simple chemical synthetic protocol. Boronic acid is capable of making covalent bonds between its diol group and the *cis*-diol group of the carbohydrate chain on a HbA_{1C} surface with no additional treatment such as a catalyst, heat, or pH. Second, unreacted free FcBA molecules after completion of the reaction were separated from HbA_{1C}-FcBA conjugates using ultrafiltration. Third, the separated portion of the free FcBA, or the purified/concentrated portion of the HbA_{1C}-FcBA conjugate, was electrochemically analyzed.

From the biospecific interaction between boronic acid and carbohydrate from glycated proteins, the FcBA-tagged HbA_{1C} was produced. The molecular weights of HbA_{1C} and FcBA are 64,000 to 65,000 daltons and 229.86 daltons, respectively. Therefore, we selected an ultrafiltration membrane having a molecular weight cut-off (MWCO) of 30,000 in order to retain the HbA_{1C}-FcBA conjugate and separate the unreacted free FcBA. Because the conjugation reac-

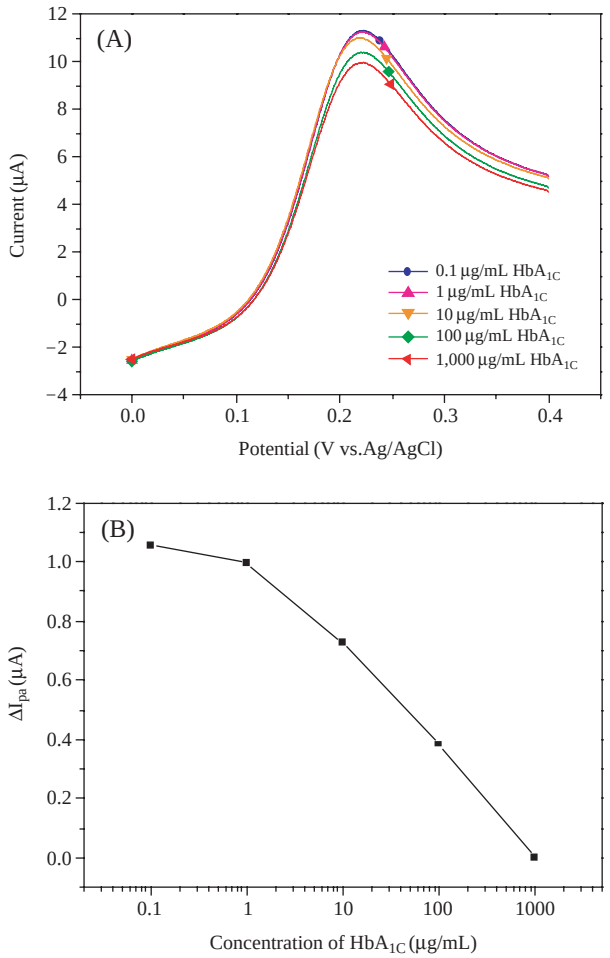


Figure 3. Electrochemical analysis of the free (unreacted) FcBA. (A) Voltammetric results from different HbA_{1C} concentrations. The concentration of FcBA was fixed at 0.5 mM. (circle) 0.1 μg/mL HbA_{1C}, (up triangle) 1 μg/mL HbA_{1C}, (down triangle) 10 μg/mL HbA_{1C}, (diamond) 100 μg/mL HbA_{1C}, (left triangle) 1,000 μg/mL HbA_{1C}, (B) Background-subtracted anodic peak currents from respective voltammograms.

tion was so simple, of which the reaction mixture consisted of only FcBA and HbA_{1C} without any additional chemicals or treatment, the single-step purification by ultrafiltration was sufficient.

For the measurement of HbA_{1C} concentration in the reaction sample, the unreacted free FcBA was first chosen as the analyte because it was easy to be collected and is directly applicable to electroanalysis. However, a drawback of this method is that the detection of the unreacted portion of the tagging molecule produced a calibration curve exhibiting a reciprocal proportionality to the target HbA_{1C} concentration with a significant background signal level. Figure 3(A) shows the cyclic voltammograms under the 0 to 0.4 V potential window, according to the HbA_{1C} concentration. The calibration curve of Figure 3(B) was plotted using sampled data at anodic peak current (I_{pa}) from the respective voltammograms. The voltammograms exhibited a decreasing peak current but with a fixed peak potential in the pattern. As the concentration of HbA_{1C} increased, the number of FcBA tagged on HbA_{1C} was also raised, suggesting that the concentration of unreacted free FcBA had decreased. Figure 3(B) showed that the electrochemical signal from the unreacted FcBA decreased as the concentration of HbA_{1C} increased, supporting the notion that the intended reactions took place successfully. From these results, we confirmed that this system is usable to detect HbA_{1C} in solution, and that the estimated detection range is from 0.1 to 1,000 μg/mL. However, the observed inverse proportionality of the signals to the concentration of the target molecule has an adverse effect on sensitivity due to the relatively large background current. In order to overcome this weak point, we moved on to the detection of the HbA_{1C}-FcBA conjugates in order to obtain a direct proportion of signal to target molecule.

Measurement of HbA_{1C} through the Detection of HbA_{1C}-FcBA Conjugates

Subsequently, we carried out an electrochemical measurement of HbA_{1C} concentration based on the HbA_{1C}-FcBA conjugates. The FcBA was conjugated with HbA_{1C}, and the resulting conjugate was purified using ultrafiltration. Then, the concentration of HbA_{1C} -FcBA conjugates in retentate was readjusted for the electroanalysis step, ranging from 0.1 to 1,000 μg/mL of HbA_{1C}. Samples containing different concentrations of HbA_{1C}-FcBA conjugate were analyzed by cyclic voltammetry. As shown in Figure 4(A), anodic currents increased in proportion to the HbA_{1C} concentration under the 0 to 0.4 V potential window, confirming that the target HbA_{1C} was detectable in this assay mode. A calibration curve was also drawn by using anodic peak currents (I_{pa}) from respective voltammograms (Figure 4(B)). However, the I_{pa} difference obtained between the maximum and minimum HbA_{1C} concentration was only 0.4 μA. This observation could be explained by the limited amount of FcBA that generates the sensor signal. It is assumed that the number of FcBA molecules that can be bound to an HbA_{1C} molecule is very limited because there is only one binding site at each N-terminal of the dual β-chain of HbA_{1C}. With the current signal level, therefore, the sensitivity was insufficient for a useful HbA_{1C} biosensor. To make up for the low sensitivity, a signal amplification strategy using GOX-mediated bioelectrocatalysis was employed.

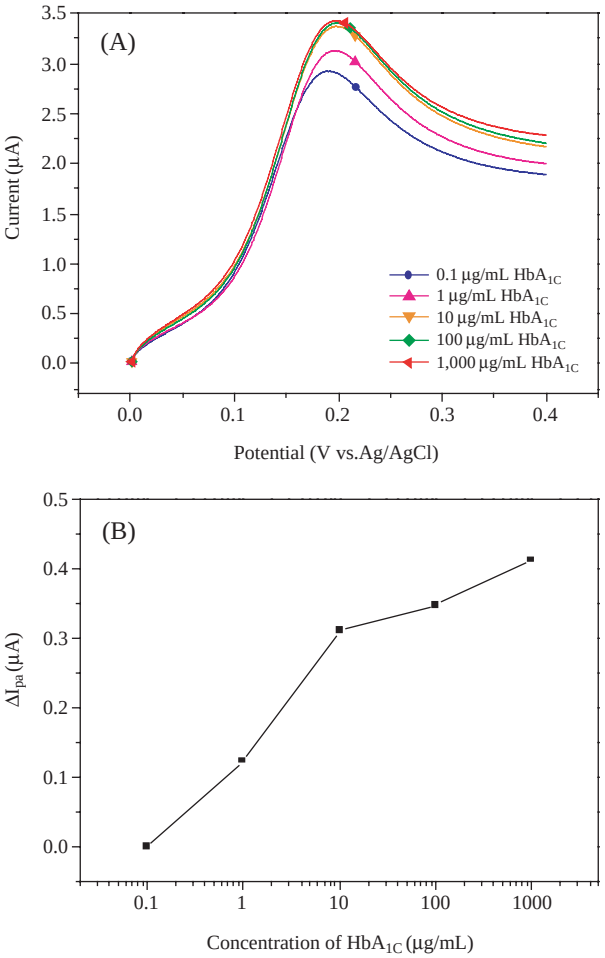


Figure 4. Electrochemical analysis of the HbA_{1C}-FcBA conjugate. (A) Voltammetric traces registered from samples with different HbA_{1C} concentrations. (circle) 0.1 μg/mL HbA_{1C}, (up triangle) 1 μg/mL HbA_{1C}, (down triangle) 10 μg/mL HbA_{1C}, (diamond) 100 μg/mL HbA_{1C}, (left triangle) 1,000 μg/mL HbA_{1C}, (B) Background-subtracted anodic peak currents from respective voltammograms.

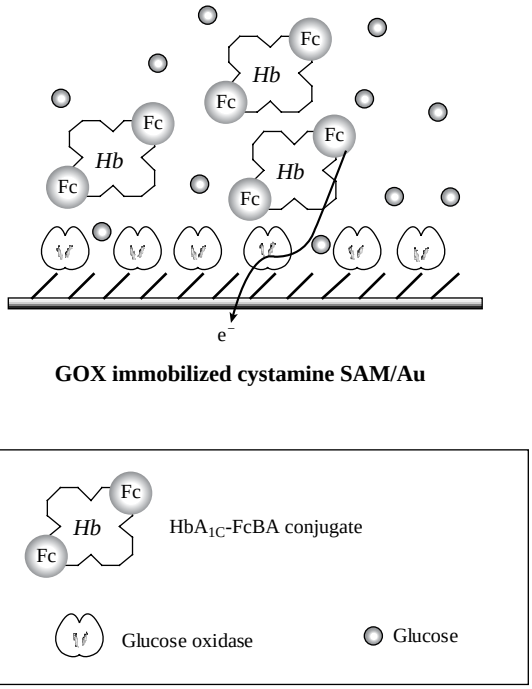


Figure 5. Schematic representation of the bio-electrocatalyzed signal amplification using a GOX-modified electrode. The electrolyte solutions used in the experiment contain HbA_{1C}-FcBA conjugates and 10 mM glucose.

Signal Amplification Using GOX-mediated Bioelectrocatalysis

We have developed an electrochemical signaling method with bioelectrocatalyzed enzymatic signal amplification. GOX was chosen to amplify the electrochemical signal by its enzymatic catalysis because FcBA could behave as an electron-transferring mediator for this enzyme. To maintain the physiological activity of the immobilized GOX on the gold electrode and to register the amplified signal efficiently, the self-assembled monolayer technique was used. A cystamine self-assembled monolayer was prepared to provide surface amine groups for the GOX immobilization. GOX was immobilized to the electrode surface after periodate oxidation, which changed the enzyme amine-reactive. With the GOX-modified electrode, the HbA_{1C}-FcBA conjugate was able to work as an electron-transferring mediator in the presence of glucose. By measuring the bioelectrocatalyzed signal from the electrodes, the concentration of target HbA_{1C} could be analyzed with a higher sensitivity. This signaling strategy is schematically shown in Figure 5.

Figure 6(A) compares the voltammetric results from the bioelectrocatalyzed signal amplification.

In the presence of non-labeled HbA_{1C} only, no distinct redox currents were observed from the GOX-modified electrode (up triangle). In comparison, typical redox peaks were registered when the HbA_{1C}-FcBA conjugate was added in the electrolyte (square). With the addition of 10 mM of glucose in the solution containing the HbA_{1C}-FcBA conjugate, a typical cyclic voltammogram for the GOX bioelectrocatalysis was found (circle). The elevation of the anodic current and the diminished cathodic peak in the voltammogram proved the desired bioelectrocatalytic reaction. As a control, with the electrolyte containing non-labeled HbA_{1C} and glucose, an irreversible voltammogram was registered, showing the production and

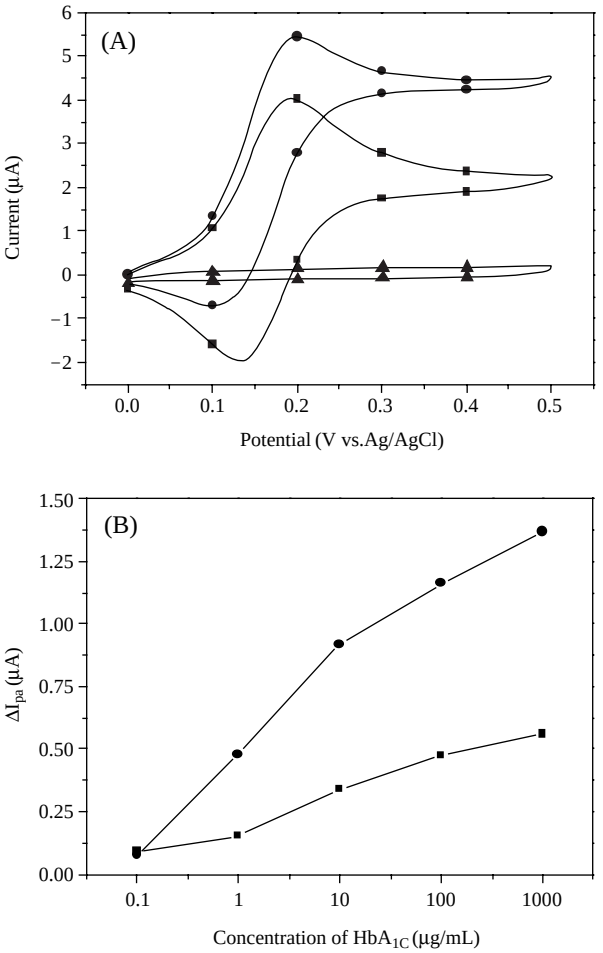


Figure 6. Bio-electrocatalyzed signal amplification using a GOX-modified electrode. (A) Cyclic voltammograms from different solution conditions: in the presence of HbA_{1C}, non-labeled (up triangle), in the presence of HbA_{1C}-FcBA conjugate and without glucose (square), and in the presence of HbA_{1C}-FcBA conjugate and 10 mM glucose (circle). (B) Signal comparisons according to the concentration of HbA_{1C}. Calibrations from amplified mode (circle) and unamplified mode (square) are shown.

anodic oxidation of hydrogen peroxide (data not shown). From these voltammetric observations, we confirmed that the signal amplification method worked effectively. Also, by comparing the calibration curves for HbA_{1C} through the enzyme-mediated signal amplification and unamplified methods as shown in Figure 6(B), we proved a signal and sensitivity enhancement by bioelectrocatalysis.

In conclusion, as an important target analyte for long-term diabetes control and treatment, glycated hemoglobin, HbA_{1C}, was analyzed electrochemically in this study. FcBA was employed as the targeting molecule, having both a biospecific binding capability to glycated proteins and electrochemical activity for signal registration. Furthermore, an enzyme-mediated signal amplification strategy was conducted to improve the biosensor sensitivity. With the results, it is not only expected that HbA_{1C} can be analyzed, but also a variety of glycoprotein that contain peripheral sugar group can be applied.

Materials and Methods

Materials

Glycated hemoglobin (HbA_{1C}), purchased from Fluka, was used without further purification. Ferroceneboronic acid (FcBA) and cystamine were acquired from Aldrich. Glucose oxidase (GOX) and D-(+)-glucose were purchased from Sigma. Absolute ethanol (EtOH, HPLC grade) was purchased from Fisher Scientific. The CENTRICON® centrifugal filter device with a 30,000 molecular weight cut-off Ultracel® YM membrane was acquired from Amicon. For the buffer solution, a phosphate-buffered saline solution containing 0.1 M phosphate and 0.15 M NaCl (PBS, pH 7.2) was prepared in doubly distilled and deionized water with a specific resistance of over 18 MΩ · cm, and was used throughout the study. All other materials and solvents used were of the highest quality available and purchased from regular sources.

Detection A: Biosensor Signaling Using the Direct Electroanalysis of FcBA and HbA_{1C}-FcBA

Samples of HbA_{1C} of predetermined concentrations (0.1 to 1,000 μg/mL) were prepared in 1.0 mL deionized water and stored at 4°C for 48 hours before use. We also made a 0.1 M stock solution of FcBA in EtOH and diluted it with a PBS buffer before use. The FcBA was mixed with HbA_{1C} samples at a ratio of 1 : 1 (v/v), and the solution was set to react for 20 min at room temperature. After the conjugation reaction, a CENTRICON filtration setup was used, containing a 30,000 MWCO membrane. An Eppendorf centrifuge 5810R was used to provide a centrifugal force of 2,465 g for the filtration step. For the electrochemical analysis, two types of reaction products were employed. One was unreacted free FcBA from the conjugation reaction that was able to pass through the filter membrane. The other analyte was the HbA_{1C}-FcBA conjugate, which was purified and concentrated further. Free and unreacted FcBA was first chosen as the analyte because it was easy to be collected. For the analysis with HbA_{1C}-FcBA conjugates, the sample recollection and concentration readjustment steps should be added.

Voltammetric measurements were conducted using an electrochemical analyzer model 630B (CH Instruments) connected to a laptop computer. A standard three-electrode configuration with an evaporated gold working electrode, a platinum auxiliary electrode, and an external Ag/AgCl reference electrode were utilized. The electrolyte solutions including free FcBA or HbA_{1C}-FcBA conjugates were analyzed using cyclic voltammetry, under a potential scan rate of 5 mV/s.

Detection B: Biosensor Signaling Using GOX-mediated Signal Amplification

The electrolyte solutions used in the Detection B experiment were the same as the HbA_{1C}-FcBA conjugate in Detection A. Electrochemical signaling was conducted to register the current from the GOX bioelectrocatalysis at the enzyme-modified gold electrodes (Figure 5). In order to fabricate the platform self-assembled monolayer on the gold electrodes, freshly evaporated thin-film gold surfaces were prepared via the resistive evaporation of 200 nm of Au (99.999%) onto titanium-printed (20 nm Ti) Si[100] wafers. Prior to the construction process, the evaporated gold surfaces were cleaned by immersion in a piranha solution containing H_2SO_4/H_2O_2 (4 : 1, v/v) for 5 min. (*Caution*: the piranha solution reacts violently with most organic materials and must be handled with extreme care.) After cleaning the electrodes completely with DDW three times, the gold surfaces were amine-modified by immersing the chip in a solution of 5 mM cystamine in DDW for 20 min. Then, the electrodes were rinsed in DDW and PBS. For the immobilization of GOX onto the amine-activated surface, GOX was oxidized by sodium periodate. One milligram of GOX was reacted for 1 hr with 1.5 mg of sodium periodate in 1 mL of PBS in darkness at 4°C. Oxidized GOX was purified via an ultrafiltration membrane with a MWCO of 30,000 CENTRICON and finally concentrated to 1 μg/mL in PBS. The systamine/gold surface was modified with the signaling enzyme by dipping it in a 40 μM periodate-oxidized GOX solution for 30 min. The GOX-functionalized electrode was used to detect the electrochemical signal. Voltammetric measurements were conducted in the presence of the HbA_{1C}-FcBA conjugate as an electron-transferring mediator and the 10 mM glucose substrate under a potential sweep rate of 5 mV/s.

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