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Zinc Ion-Mediated Concentration of Glycated Hemoglobin for Electrochemical Biosensing

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Abstract

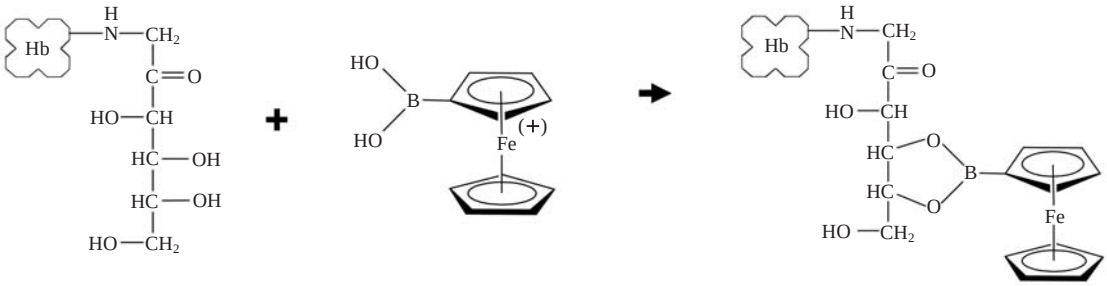
We developed a biosensing strategy for glycated proteins in red blood cells. Glycated hemoglobin (HbA_{1C}) is recognized as an important target molecule to improve the control and longterm treatment of diabetes mellitus. We employed ferrocene boronic acid (FcBA) as a signaling molecule for electrochemical biosensing, since it possesses both signaling capability and biospecificity toward the target glycated proteins. FcBA was reacted with samples containing HbA_{1C}, and the resulting conjugate was purified and highly concentrated through treatment with zinc (Zn) ions. Zn binds to hemoglobin with an association constant of 1.3×10^7 M^{-1}. By employing Zn-mediated concentration/separation, we were able to purify HbA_{1C} easily and rapidly. After the solution-phase purification and concentration step, electrochemical signaling was performed for HbA_{1C}. Using cyclic voltammetry, the dynamic detection range obtained for HbA_{1C} was around 3-15% (HbA_{1C} per total hemoglobin), which covered the clinically important range. This technology could be implemented in a diagnostic biosensor and would be applicable to other glycoproteins that need to be analyzed.

Keywords: Glycated proteins, HbA_{1C}, Hemoglobin, Zinc ion, Electrochemical biosensor

Introduction

Glycated hemoglobin (Hemoglobin-A_{1C}, HbA_{1C}) is examined as a potential target molecule for the detection and management of diabetes mellitus[1]. To detect this molecule, the hemoglobin portion in the human red blood cell should be collected and analyzed. Adult human hemoglobin is mainly composed of HbA. The remainder is composed of HbA_2 (2.5%), HbF (0.5%), HbA_{1a+1b} (1 to 2%), and HbA_{1C} (3 to 6%)[2]. HbA_{1C} could be used as a molecule to indicate the progression of diabetes. HbA_{1C} is formed by a non-enzymatic reaction between glucose and the N-terminal valine of the hemoglobin chain at a constant slow rate throughout the lifetime of a red blood cell. The determination of HbA_{1C} helps in the monitoring of the long-term progression of diabetes without the influence of short-term fluctuations in the blood glucose level, because the lifespan of an erythrocyte (red blood cell) is relatively long (100-120 days). Thus, it is considered that this molecule can be applied in the control and management of diabetes. HbA_{1C} is expressed as a percentage of HbA_{1C} per total hemoglobin, with 4 to 6% considered normal. This corresponds to a blood sugar range of about 60 mg/dL to about 120 mg/dL in healthy people. The American Diabetes Association recommends an HbA_{1C} of 7% (150 mg/dL) or lower as the guideline. The detection principle for HbA_{1C} is based on its unique molecular feature. HbA_{1C} is a glycoprotein with a carbohydrate moiety on its three dimensional molecular surface; this moiety can be employed as the targeting site[3-6]. In order to capture and detect glycoproteins, we need an anchor molecule that can selectively bind to the target carbohydrates. It has been reported that the boronic acid group has an affinity to the carbohydrate, forming a covalent bond between its diol group and *cis*-diols from carbohydrates[7,8]. We utilized ferroceneboronic acid (FcBA) for developing a biosensing application that exploits this specific reaction between boronic acid and glycoproteins (HbA_{1C})[9,10]. FcBA is appropriate since it contains both boronic acid and ferrocenyl groups, possesses electrochemical activity, and can play a role in enzyme-catalyzed signaling[11]. 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 by electrochemical methods.

Metal ions such as the Zn ion interact with proteins via mechanisms influenced by the metal type and degree of coordination. A series of enzymes possess binding sites specific to Zn ions. X-ray crystallographic analysis reveals that the Zn ion-binding amino acids are histidine, cysteine, glutamic acid, and aspartic acid[12]. Zn ions also interact with hemoglobin[13-15]. Many studies have investigated this interaction because it improves the oxygen affinity of sickle cells.



HbA_{1C} | Ferrocene/ferrocenium boronic acid (FcBA) | HbA_{1C}-FcBA conjugate

Figure 1. Mode of specific covalent bonding between HbA_{1C} and ferrocene/ferrocenium boronic acid (FcBA).

The Zn ion actively binds with cysteine-93 (sulfhydryl group) and histidine-143 of the hemoglobin beta chain and, to a lesser extent, with histidine-146 and -97 and aspartic acid-94. In addition, one Zn ion binds with two heme groups; the binding constant between hemoglobin and Zn ions is $1.3 \times 10^7\ M^{-1}$. The binding reaction causes the reorientation of hemoglobin molecules[16]. This conformational change induces selective hemoglobin coagulation and allows purification. Zn-mediated purification could be used specifically for purifying HbA_{1C}-FcBA. In this study, an effective electrochemical biosensing method for HbA_{1C} was developed by using a Zn ion-mediated concentration and purification.

Results and Discussion

Verification of HbA_{1C}-FcBA Conjugates Formation

HbA_{1C} was determined as an index for the diagnosis and management of diabetes. The *cis*-diol groups of the carbohydrate chain on the surface of HbA_{1C} can form covalent bonds specifically with boronic acid. FcBA, which possesses electrochemical activity through its ferrocenyl group and specificity with glycated proteins, was selected as the signaling molecule. The conjugate between HbA_{1C} and FcBA was separated by the dialysis of the reaction mixture. A semi-permeable membrane allows free FcBA to pass through it by diffusion, but the HbA_{1C}-FcBA cannot pass because the protein conjugate is too large in size. Figure 2A shows the electrochemistry of FcBA, which exhibits redox peaks around 200 mV vs. Ag/AgCl reference electrode. The cyclic voltammogram shown in Figure 2B revealed the presence of FcBA interacting with HbA_{1C}, thereby indicating the occurrence of a combination between HbA_{1C} and FcBA. The voltammogram in Figure 2C shows bioelectrocatalytic signal amplification in the presence of glucose oxidase (GOX) and glucose. GOX amplified the electrochemical signal by its enzymatic catalysis because FcBA could behave as an electron-transferring mediator for this enzyme[17]. From the result, we found that the ferrocenyl groups bound to the HbA_{1C} molecules retained the electrochemical activity, enabling the designed signaling strategy.

Zinc-mediated Purification and Analysis of HbA_{1C} through HbA_{1C}-FcBA Conjugates

The HbA_{1C}-FcBA conjugate, which had been purified using Zn, was detected electrochemically. Zn-mediated purification was performed using a mini-centrifuge, and a short precipitation time of 30 s was required (Figure 3). Zn ions interacted with hemoglobin with a significantly high binding constant of $1.3 \times 10^7\ M^{-1}$, enabling specific purification of hemoglobin. When the Zn ions came in contact with the HbA_{1C}-FcBA conjugates, the Zn ions and hemoglobin beta chains combined and precipitated by the protein coagulation. As shown in Figure 3, this process was accelerated by a slight centrifugation and the hemoglobin precipitate could be collected. Before precipitation, the HbA_{1C}-FcBA conjugates were evenly distributed in the solution. The HbA_{1C}-FcBA conjugates combined with Zn ions and coagulated along the direction of the centrifugal force. The selective separation of hemoglobin via Zn ions was confirmed from the results. By using HbA_{1C}-FcBA samples of different concentration, Zn ion-mediated separation followed by electrochemical tests was conducted. Figure 4A shows voltammograms over potential windows of 0.1 to 0.5 V vs. Ag/AgCl. Increasing the HbA_{1C} concentration enhanced the signal proportionally. From the results, we confirmed that the Zn ion-mediated purification system can be effectively used to detect HbA_{1C} in solution. Figure 4B shows that the electrochemical signal from the HbA_{1C}-FcBA conjugate increased with the HbA_{1C} concentration. The clear differences between the sig-

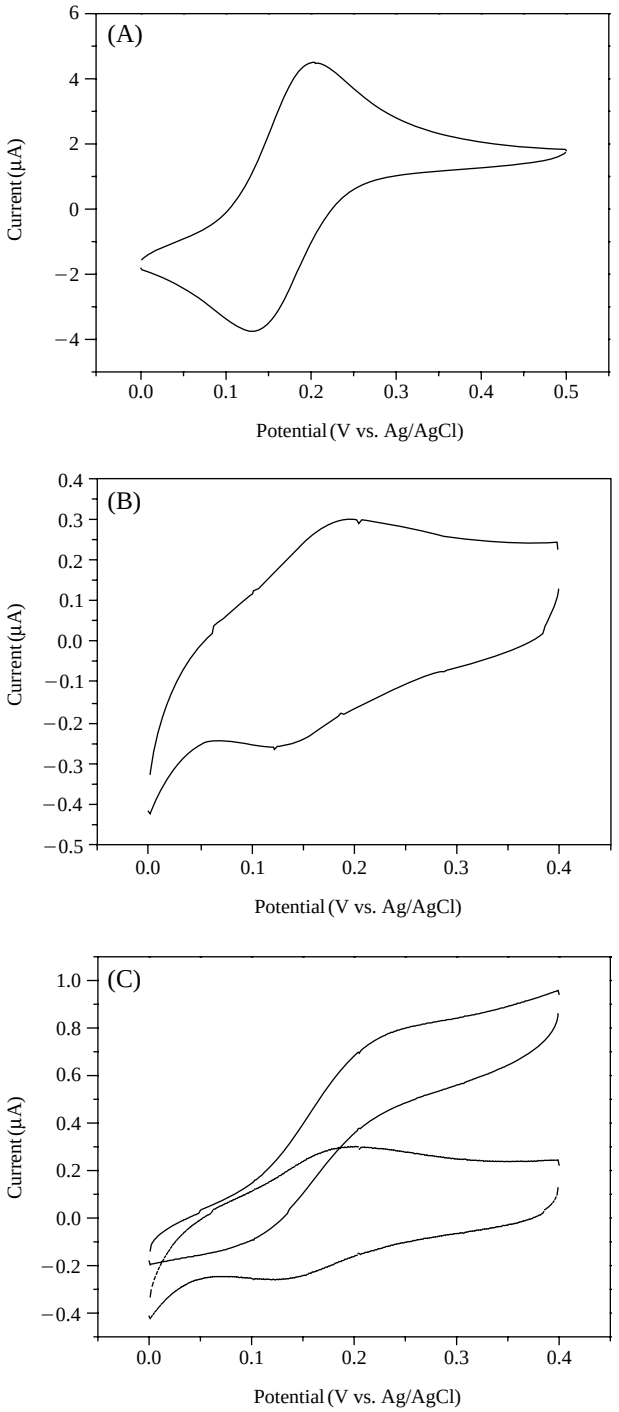


Figure 2. Cyclic voltammograms of FcBA and HbA_{1C}-FcBA conjugate. (A) 0.1 mM FcBA, (B) separated HbA_{1C}-FcBA conjugate after dialysis for 20 h, (C) bio-electrocatalyzed signal amplification using 0.05 mg/mL GOX and 10 mM glucose in the presence of HbA_{1C}-FcBA conjugate.

nals corresponding to various concentrations of HbA_{1C} enable us to verify that the intended reactions occurred

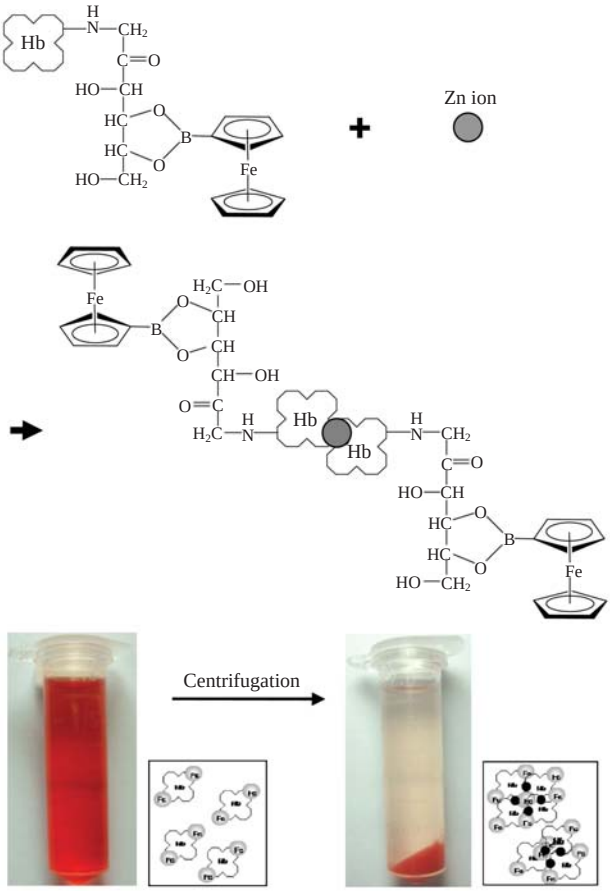


Figure 3. Zinc ion-mediated concentration and purification of HbA_{1C} conjugates. Schematic representation of Zn ion-mediated aggregate formation (Upper panel). Pictures showing the process are also included (Bottom panel).

successfully. The estimated detection range was 0.125 to 1 mg/mL.

Preparation of HbA_0 for the Analysis of $\%HbA_{1c}$

Normal blood hemoglobin consists of both HbA_{1C} and HbA_0. For the diagnosis of diabetes, the percentile concentration ($\%HbA_{1C}$ per total hemoglobin) is employed as the standard unit of HbA_{1C}. To ensure the accuracy of the HbA_{1C} sample, non-glycated hemoglobin (HbA_0) was chemically prepared and used (*See Materials and Methods Section*). A structural feature of HbA_{1C} is the presence of a sugar moiety at the NH_2-terminal valine of the beta chain. To block the *cis*-diol group, the carbohydrate chains of HbA_{1C} were oxidized by sodium periodate, and the resulting carbaldehydes were reacted with L-lysine. Then, the conjugate was reduced by sodium cyanoborohydride to stabilize the combination. The electrochemical signal of the

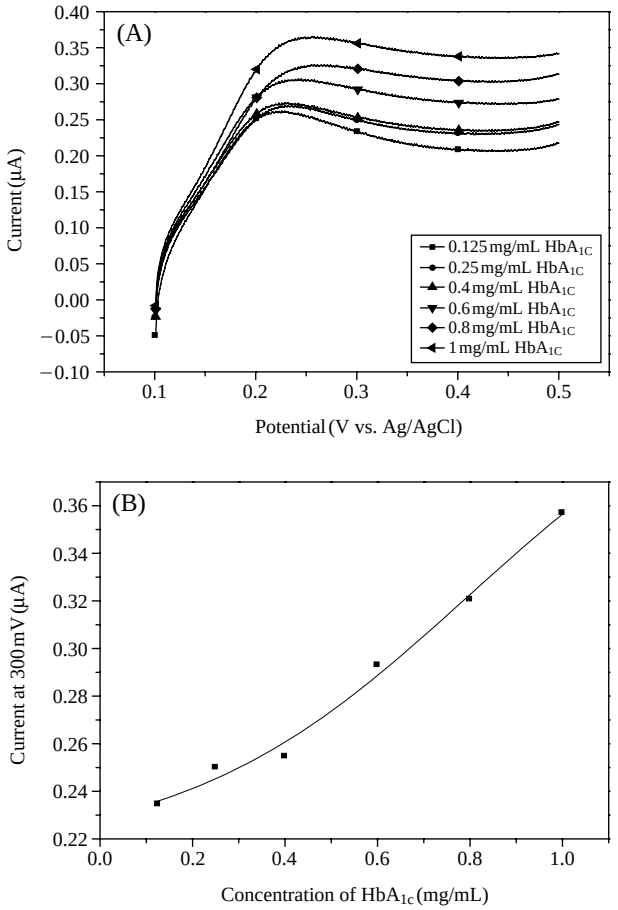


Figure 4. Electrochemical analysis of the HbA_{1C}-FcBA conjugate purified using Zn ions. (A) Voltammetric traces registered from samples with different HbA_{1C} concentrations: (square) 0.125 mg/mL HbA_{1C}, (circle) 0.25 mg/mL HbA_{1C}, (up triangle) 0.4 mg/mL HbA_{1C}, (down triangle) 0.6 mg/mL HbA_{1C}, (diamond) 0.8 mg/mL HbA_{1C}, (left triangle) 1 mg/mL HbA_{1C}. (B) Signals were registered from the anodic currents at 300 mV in the cyclic voltammograms for each experiment.

manufactured HbA_0 was compared with the signals of HbA_{1C} and the HbA_{1C}-FcBA conjugate. As shown in Figure 5, pure HbA_{1C} did not produce an anodic current between 0.1 and 0.5 V vs. Ag/AgCl, whereas the HbA_{1C}-FcBA conjugate clearly produced an anodic current at 200 mV, owing to the presence of the ferrocenyl group. HbA_0 was mixed with FcBA using the same conjugate process as that used for the HbA_{1C}-FcBA conjugate. The electrochemical signal of the mixture was much weaker than that of the HbA_{1C}-FcBA conjugate. This result indicates that even though the carbohydrate chain of HbA_{1C} was not blocked perfectly, the chemically prepared HbA_0 can be used for the sample preparation and detection of the $\%HbA_{1C}$.

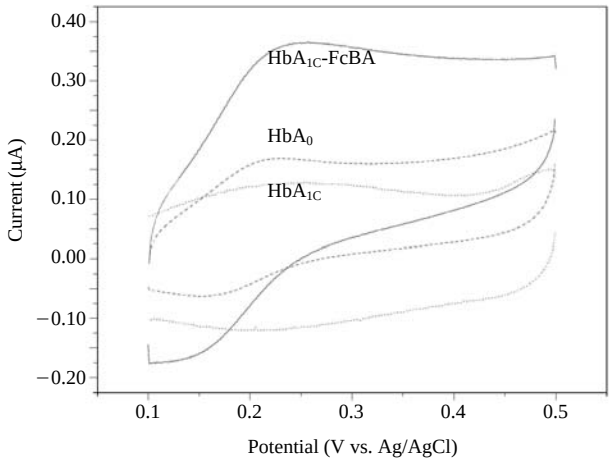


Figure 5. Comparison of cyclic voltammograms of HbA_{1C}-FcBA, HbA_{1C}, and HbA_0.

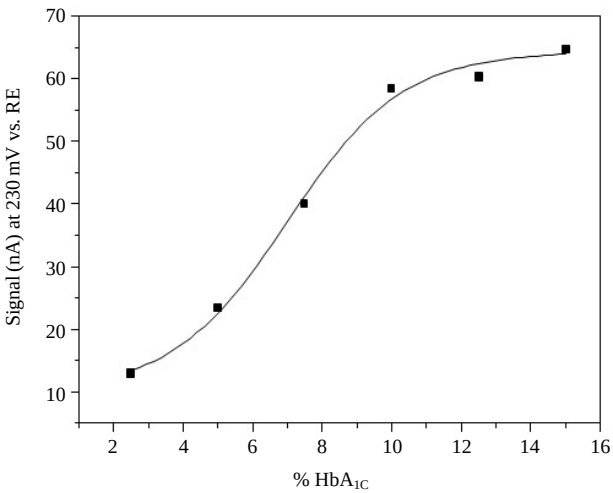


Figure 6. Electrochemical analysis of the percentage of HbA_{1C} purified with Zn. Signals were registered from the anodic currents at 230 mV in the cyclic voltammograms for each experiment.

Electrochemical Biosensing of $\%HbA_{1C}$

In general, it is considered that a $\%HbA_{1C}$ of 4 to 6% is normal, while 7% indicates the occurrence of diabetes. HbA_{1C} and chemically manufactured HbA_0 were mixed in different component ratios, and the total hemoglobin was identified as a constant concentration. HbA_{1C} concentrations ranging from 2.5 to 15% were conjugated with a fixed concentration of FcBA, and the HbA_{1C}-FcBA conjugates were purified using Zn ions. Each of these HbA_{1C}-FcBA conjugates was analyzed by cyclic voltammetry. The results show that the magnitude of the signal increased with

%HbA_{1C} and that the signals before and after 7% HbA_{1C} could be distinguished. From the respective cyclic voltammograms, a calibration curve was drawn using the anodic currents at 230 mV vs. Ag/AgCl (Figure 6). From the calibration, we found that %HbA_{1C} values in range of 2.5-15% can be detected by employing the method; this detection range covers the reference range for the clinical diagnosis of diabetes. There results confirmed that the HbA_{1C} test from this study could be used to diagnose and control diabetes.

Conclusions

We developed an electrochemical biosensing method for the assessment of HbA_{1C}; this method assesses the average blood glucose level over a period of 2-3 months. To perform the HbA_{1C} measurements, FcBA was conjugated with HbA_{1C}. The HbA_{1C}-FcBA conjugate was then separated from the unreacted free FcBA using Zn ions. Zn ions specifically interact with hemoglobin, and the process is accompanied by protein coagulation. This Zn-mediated purification method was capable of analyzing HbA_{1C} concentrations of between 2.5 and 15%; furthermore, miniaturization of the purification process could be achieved. The results showed that signals corresponding to HbA_{1C} concentrations of between 2.5 and 15% could be distinguished and confirmed that diabetes could be detected by using this assay.

Materials and Methods

Materials

For our experiments, HbA_{1C} was purchased from Fluka and used without further purification. FcBA and sodium cyanoborohydrite were acquired from Aldrich. L-Lysine, Zinc chloride ($ZnCl_2$) and sodium periodate were acquired from Sigma-Aldrich. Absolute ethanol (EtOH, HPLC grade) was obtained from Fisher Scientific, and SnakeSkin® Pleated Dialysis Tubing was purchased from Thermo Scientific. 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 of the other materials used were of the highest quality available and purchased from regular sources.

Apparatus

Voltammetric measurements were conducted using a model 630B electrochemical analyzer (CH Instruments) connected to a laptop computer. A standard three-electrode configuration was utilized with an evaporated gold working electrode, a platinum auxiliary electrode, and an external Ag/AgCl reference electrode. A Quick Spin Minifuge (Labnet international, Inc.) was used for the precipitation step.

Zinc-mediated Purification of Glycated Hemoglobin and Detection of HbA_{1C}-FcBA Conjugates

To verify the combination of HbA_{1C} and FcBA, the HbA_{1C}-FcBA conjugate was purified using a dialysis pack (MWCO 10.000) for 20 h at 4˚C. The purified conjugate solution was stored at 4˚C. Different concentrations (0.125, 0.25, 0.4, 0.6, 0.8, and 1 mg/mL) of HbA_{1C} were prepared by diluting with deionized water, and a 32 mM stock solution of FcBA was prepared in EtOH and diluted 100 fold with a PBS buffer before use. FcBA was combined with the HbA_{1C} samples at a ratio of 1 : 1 (v/v), and the solutions were allowed to react for 20 min at room temperature. It has been known that boronate reaction occurs readily under alkaline condition. Thus, we think ferrocenium portion of FcBA reacts efficiently in the employed solution condition of pH 7.2 because the pK_a of ferrocenium-BA is around 5.8. After the conjugation reaction, we purified the HbA_{1C}-FcBA conjugate that was formed by binding with Zn ions. The HbA_{1C}-FcBA conjugate was combined with 16 mM $ZnCl_2$ at a ratio of 1 : 1 (v/v), and precipitated using a mini-centrifuge. Each sample was then washed three times and re-suspended in the PBS buffer. Voltammetric measurements were conducted in the presence of the HbA_{1C}-FcBA conjugate as an electron-transferring mediator at a potential sweep rate of 5 mV/s.

Preparation of HbA_0

HbA_{1C} exists as a percentage of human blood, along with non-glycated hemoglobin. To detect the percentage of HbA_{1C}, a chemical reaction was performed to prepare artificial HbA_0. For the preparation of non-glycated hemoglobin, HbA_0, the carbohydrate chain of HbA_{1C} was blocked. As shown in Figure 7, HbA_{1C} was linked with L-lysine by a chemical reaction. By reacting 0.23 mM of HbA_{1C} with 8.0 mM of sodium m-periodate ($NaIO_4^-$) for 1 h at 4˚C, the carbohydrate chain of HbA_{1C} was made amine-reactive. Then, oxidized HbA_{1C} sample was reacted with 2.6 mM L-lysine for 1 h at 4˚C for blocking, followed by the sodium cyanoborohydride reduction of Schiff's bases (CAUTION: Since cyanoborohydride is extremely toxic, all operations involving this compound were performed carefully in a fume hood). The resulting HbA_0 solution was purified by dialysis overnight and was stor-

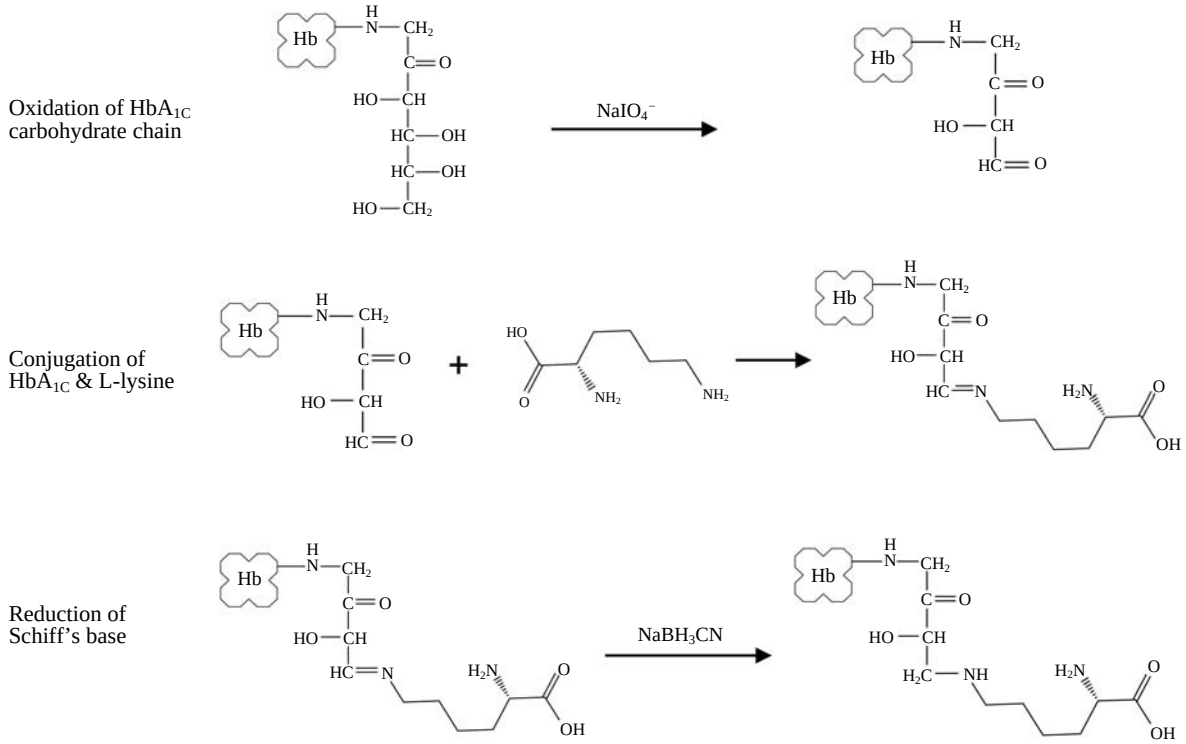


Figure 7. Schematic illustration of conjugation reaction of lysine-tagged HbA_{1C} (HbA_0).

ed at 4°C before use. Confirmatory experiments were conducted to assess whether the carbohydrate chain of HbA_{1C} had been blocked. The manufactured HbA_0 and HbA_{1C} were mixed with FcBA and purified under the same conditions as specified in the previous section. Along with the confirmatory experiments, we conducted experiments to measure the electrochemical signal for pure hemoglobin. Cyclic voltammetry was used to detect the signal for each sample at a potential scan rate of 5 mV/s.

Detection of %HbA_{1C}

We developed an electrochemical signaling method for the detection of HbA_{1C}. The first part of the experiments was the induction of a conjugate reaction between the target molecule and FcBA, followed by the separation of the reaction products by using Zn ions. The target molecule HbA_{1C} was conjugated with FcBA by a simple chemical synthetic protocol without further treatment. The *cis*-diol group of the carbohydrate chain on the HbA_{1C} surface formed a covalent bond with the diol group of boronic acid. The HbA_{1C} test results are expressed as a percentage, with 7% or above considered to indicate the occurrence of diabetes. To detect HbA_{1C} concentrations of around 7%, HbA_{1C} concentrations ranging from 2.5% to 15% were prepared. For this purpose, HbA_{1C} and HbA_0, prepared using the procedure described above, were mixed with different component ratios. Various percentages of HbA_{1C} and 0.32 mM FcBA were reacted at a ratio of 1 : 1 (v/v) to form HbA_{1C}-FcBA conjugates. Subsequently, these HbA_{1C}-FcBA conjugates were purified using Zn ions. $ZnCl_2$ (16 mM) was mixed with the HbA_{1C} and FcBA complex at a ratio of 1 : 1 (v/v). The precipitate was gathered using a mini-centrifuge and washed three times with PBS buffer so as to separate the HbA_{1C}-FcBA conjugate from free FcBA. The last part of the experiments was the detection of the HbA_{1C}-FcBA conjugate through an electrochemical method. Each of the HbA_{1C}-FcBA conjugate samples was analyzed using cyclic voltammetry at a potential scan rate of 5 mV/s.

Acknowledgements

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