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Electrochemical Assay for Glycated Hemoglobin based on the Magnetic Particle-supported Concentration Coupled to Boronate-diol Interactions

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The separation of a specific target protein from a complex biochemical sample is important in many areas of analytical sciences. Researchers are investigating new and effective separation methods because the separation process significantly affects the price of production. The latest separation technology using a magnetic field has received much attention due to its diverse applications and because it simplifies the adsorption and separation steps.[1] This method has several advantages, including speed, simplicity, and excellent selectivity. Both small (50 ~ 200 nm) and large (1 μm and above) magnetic particles (MPs) can be used in biomolecular separation. A superparamagnetic particle has the unique and important property of exhibiting magnetism only when under a magnetic field. This means that the target molecule readily returns to a fluid medium after the magnetic separation, maintaining its initial biomolecular activity and enabling further treatments with ease.[2] Applications that are currently being studied with MPs include separation, stabilization, detection, and molecular delivery.[3] The linkage of MPs and biomolecules can be accomplished by introducing reactive groups, which biospecifically interact with the target molecules, onto the MP surface. Among several biospecific reactive groups, metal cation reacts and binds to the specific proteins. Binding of specific amino acid residues to free coordination site of metal cation affects to polypeptide folding and induces conformational change of proteins.[4] These metal cation-protein binding properties have been used in protein purification, for example, in the immobilized metal cation affinity chromatography (IMAC).[5] Especially, zinc cation exhibits affinity with hemoglobin and binds to specific histidine and cysteine residues of the hemoglobin structure.[6] The zinc-hemoglobin interaction induces conformational changes of the hemoglobin that induces coagulation.[7,8] Frantzen group demonstrated that zinc cations were able to selectively precipitate hemoglobin from whole blood lysate by controlling the hemoglobin/zinc cation ratio.[8]

As a subtype of hemoglobin, glycated hemoglobin (HbA_{1C}) is a widely used marker for long-term treatment and control of diabetes mellitus.[9] HbA_{1C} is formed when an amino terminal of the hemoglobin β chain is glycated under conditions of uncontrolled high blood glucose concentration.[10] Glucose binds nonenzymatically to hemoglobin slowly (about 0.05% per day) and indicates the average glucose level approximately 120 days prior to measurement, which corresponds to the lifetime of red blood cells.

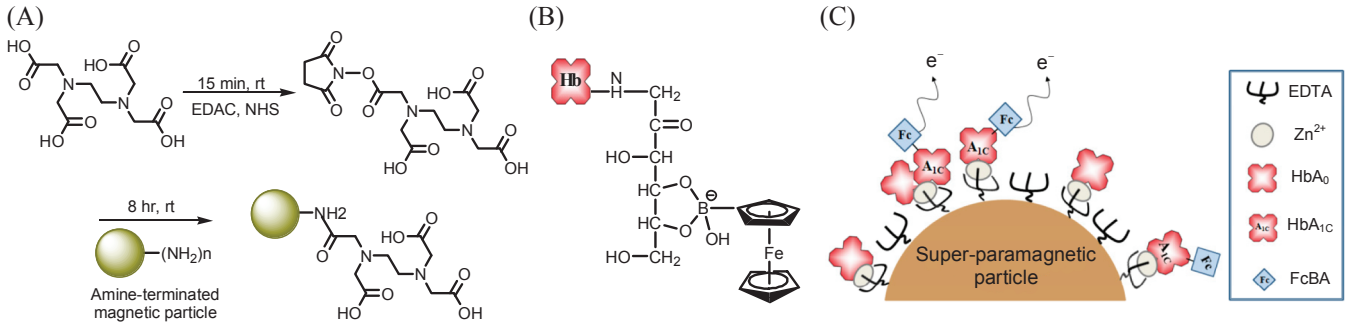
Various methodologies have been applied for HbA_{1C} detection, including boronate affinity chromatography, immunoassay, ion-exchange chromatography, colorimetry, and mass spectroscopy.[11] However, these approaches necessitate complex instrumentations and operation procedures. We have employed the electrochemical approach to evaluate the levels of HbA_{1C} to overcome these disadvantages. For the electrochemical detection, the carbohydrate moiety from the molecular surface of HbA_{1C} can be employed as the targeting site.[12]

The carbohydrate of HbA_{1C} β chain is reacted with boronate compounds containing electroactive groups, resulting in the formation of boronate-diol complexes.[13,14] Additionally, to achieve an accurate detection of HbA_{1C}, the hemoglobin separation steps are required. With unnecessary proteins such as albumins, antibodies and enzymes in hemolysates, these could act as signal interferences in the electrochemical detection of glycated hemoglobin. In this study, a zinc-modified superparamagnetic MP (ZnMP) was introduced to concentrate and purify HbA_{1C} efficiently. As a zinc chelating ligand, ethylene diamine tetraacetic acid (EDTA) was introduced to modify zinc cations to the surface of the MP. As shown by previous reports, EDTA could be covalently immobilized to amine-modified solid surface.[15-17] Under the condition of the suitable conformation, EDTA can be immobilized onto the amine-modified solid surface maintaining its chelating capability to zinc cations *in situ*.[16,17]

With the aid of ZnMP, an electrochemical assay using ferrocene boronate (FcBA) as a signaling tag was developed for HbA_{1C}. FcBA is pertinent to the signal registration according to the concentration of HbA_{1C}, because the FcBA molecule contains both diol-reactive boronic acid and electroactive ferrocenyl groups. Also, by using permanent magnet, ZnMP-HbA_{1C}-FcBA conjugates could be magnetically deposited and concentrated to the surface of a working electrode, providing signaling of amplified electrochemical currents. Details are reported herein.

Results and Discussion

The key purpose of this study is to discover an effective method to purify the HbA_{1C}-FcBA conjugate from unreacted FcBA for precise electroanalysis of HbA_{1C}. Typically, centrifugation and filtration have been used to remove particles from fluid. However, these methods are time-consuming and complex, and require specific equipment such as filters and



Scheme 1. Schematic illustrations of (A) the preparation steps of EDTA-terminated magnetic particles, (B) the chemical structure of conjugation adduct between HbA_{1C} and FcBA, and (C) the MP-assisted concentration of target HbA_{1C} and electrochemical signaling with ZnMP/HbA_{1C}-FcBA.

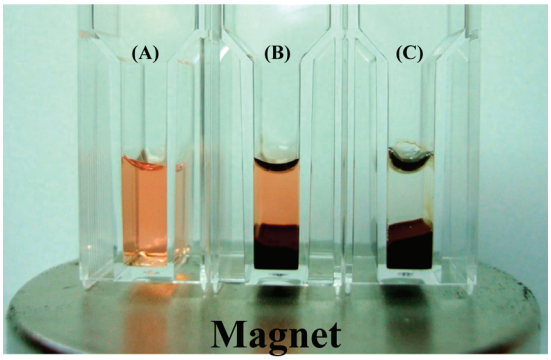


Figure 1. Comparison of three cuvettes on a permanent magnet. Samples are 1.0 mg/mL HbA_{1C} mixed with (A) phosphate buffer solution only, (B) EDTA-terminated magnetic particles, and (C) ZnMPs, respectively.

ultrafiltration systems. Magnetic separation, purification using surface-modified MPs, overcomes the weaknesses of the conventional methods. In this study, MPs have the ability to specifically bind with HbA_{1C}, once the MPs have been modified with zinc ion (ZnMP). Scheme 1 illustrates overall process of this work including the preparation of EDTA-terminated magnetic particles, the adduct from boronate-diol interaction between HbA_{1C} and FcBA and the purified product of ZnMP/HbA_{1C}-FcBA after the conjugation and purification steps. Details are in the experimental section.

After ZnMP preparation, confirmatory experiments were conducted to determine the reaction between ZnMPs and hemoglobin, including glycated hemoglobin (HbA_{1C}) and non-glycated normal hemoglobin (HbA_0). Figure 1 shows three cuvettes subjected to spectrophotometry on the magnet: the first cuvette on the left (A) contained HbA_{1C} only, and the color of the solution was red, showing even dispersal of hemoglobin molecules in solution. The middle cuvette (B) and the one on the right (C) contained hemoglobin mixed with EDTA-terminated MPs and ZnMP, respectively. Under the magnetic field, both EDTA-terminated MPs and ZnMPs were deposited at the bottom of the cuvette. In the cuvette (C), for the ZnMP treatment, the supernatant was very clear, showing that hemoglobin was collected successfully. However, in cuvette (B) for EDTA-terminated MPs, the supernatant mixture was still red in color, indicating that the hemoglobin had not successfully combined with these MPs. From these results, we could confirm that hemoglobin interacted with zinc ion on the surface of ZnMPs, while hemoglobins did not interact with the EDTA-terminated MP itself.

To confirm the reaction between ZnMPs and hemoglobin, the binding capacity of ZnMPs and hemoglobin was determined by spectrophotometry. For the experiment, 1 mg/mL of HbA_{1C} was employed as the binding target. As the concentration of ZnMPs increased (1, 5, 20, and 50 mg/mL), the binding capacity of ZnMPs with HbA_{1C} increased to 30%, 38%, 57%, and 76%, respectively. From these results, we concluded that manufactured ZnMP has the ability to capture HbA_{1C} effectively enough for use in HbA_{1C} purification. The optimum concentration of ZnMP in the reaction mixture was determined after considering the effect of ZnMP on electrochemical signaling (*vide infra*).

With the prepared ZnMP, we electrochemically analyzed HbA_{1C}. The HbA_{1C} portion in total hemoglobin was selectively reacted with FcBA *via* the cis-diol interaction, and the HbA_{1C}-FcBA conjugate was separated using ZnMPs. ZnMPs can purify all types of hemoglobin, including glycated HbA_{1C} and non-glycated HbA_0; however, FcBA interacted only with the glycated portion of HbA_{1C}. Thus, the electrochemical signal values were registered according to the concentration of HbA_{1C} based on the bound FcBA molecules. To determine the assay conditions, the HbA_{1C} sample was reacted with FcBA, and the HbA_{1C}-FcBA conjugate was analyzed after being purified with either zinc ion or various concentrations of ZnMPs. Voltammetric measurements were performed with the purified HbA_{1C}-FcBA conjugate on a gold electrode. From voltammograms, the signal current levels were registered at 400 mV *vs.* Ag/AgCl for the respective analyte concentrations. As the concentration of ZnMPs increased from 1 to 10 mg/mL, the overall electrochemical signal increased (Figure 2). The currents increased in proportion to the concentration of target HbA_{1C}, but showed different signal trends based on the concentrations of ZnMPs (1, 5, and 10 mg/mL) applied. For the condition of 1 mg/mL ZnMP in Figure 2A, a sigmoidal plot was registered according to the HbA_{1C} concentration, ranging from 0.0 to 0.6 mg/mL, after which the signal leveled off. Due to the narrow detection

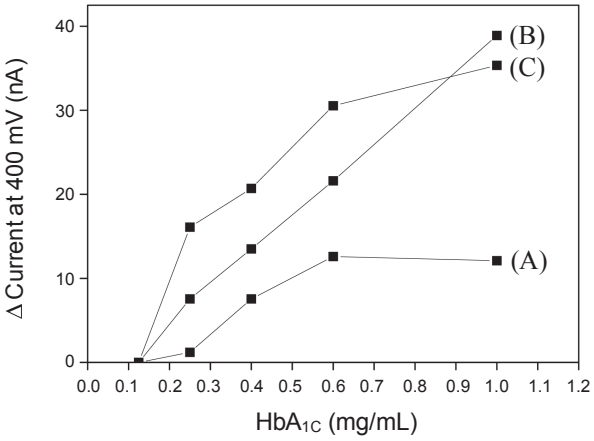


Figure 2. Electrochemical analysis of HbA_{1C} purified from ZnMPs treatment. Registration of anodic currents at 400 mV *vs*. Ag/AgCl reference electrode from respective voltammograms according to the various concentrations (0.1~1.0 mg/mL) of HbA_{1C}. The concentrations of ZnMPs are (A) 1 mg/mL, (B) 5 mg/mL, and (C) 10 mg/mL.

range and sigmoidal signal trend, the 1 mg/mL ZnMP condition was not useful. From the test with 10 mg/mL ZnMP in Figure 2C, we found that the signal increased fast but leveled off at a low HbA_{1C} concentration, which is not useful for the analysis of HbA_{1C} in the required detection range. The optimum concentration condition for electrochemical detection of HbA_{1C} was considered to be 5 mg/mL of ZnMP, since it exhibits a linear signal change between 0.0 and 1.0 mg/mL of HbA_{1C} examined (Figure 2B). Consequently, 5 mg/mL of ZnMP was chosen for detecting HbA_{1c} with the developed method. For comparison, 5 mM of zinc chloride was reacted with the HbA_{1C}-FcBA conjugate. When zinc ion was added, the coagulation process began immediately. The coagulated HbA_{1C}-FcBA conjugate was collected using a centrifuge, and the electrochemical signal was measured. Electrochemical signals of HbA_{1C}-FcBA conjugates purified with zinc ion almost corresponded to those in the ZnMP (1 mg/mL) purification process. In fact, zinc ion and ZnMPs had almost the same effect on the purification of the HbA_{1C}-FcBA conjugate, because hemoglobin purification occurred *via* the reaction between HbA_{1C} and zinc ion, either in solution or on the surface of the MPs. However, purification using ZnMPs is a faster and more convenient method than centrifugation with zinc ion in solution. Furthermore, ZnMPs capturing HbA_{1C}-FcBA conjugates could be magnetically deposited to the surface of a working electrode, enabling signaling of amplified electrochemical currents.

In the practical analysis, HbA_{1C} is measured as the percentage of glycated hemoglobin (%HbA_{1C}) per total hemoglobin (HbA_{1C} + HbA_0). The reference range for diabetes diagnosis and long-term control is 5% ~ 15% HbA_{1C}.[9,18] To ensure the accuracy of the %HbA_{1C} sample preparation, HbA_{1C} samples of different concentrations ranging from 0% to 15% were prepared by adjusting the total hemoglobin concentration to 1.5 mg/mL with HbA_0. %HbA_{1C} samples were reacted with a fixed concentration of FcBA, and the HbA_{1C}-FcBA conjugates were purified with ZnMPs. Purified ZnMP/HbA_{1C}-FcBA was electrochemically analyzed on a gold electrode with or without a permanent magnet under the working electrode. Calibration curves were drawn using anodic current differences at 400 mV from the respective cyclic voltammograms (Figure 3). We found that %HbA_{1C} values could be detected, registering the increase in current from the ferrocenyl groups bound on HbA_{1C}. However, when the ZnMP/HbA_{1C}-FcBA was dispersed and measured in the electrolyte, the signal level as well as the detection range of %HbA_{1C} was not sufficient. The observed detection range was narrow, from 0% to 6%HbA_{1C} (Figure 3B and 3C). To enhance the electrochemical signal by concentrating the conjugate at the electrolyte/electrode interface, we placed a permanent magnet under the working electrode. We could observe amplified signal levels and a wider detection range.

From the calibration with signal amplification (Figure 3A and 3C), we found that %HbA_{1C} values in the range of 0% ~ 12% can be detected, covering the reference range for the diagnosis and management of diabetes. It should also be noted that the %HbA_{1C} values in the range of 6% ~ 9%, important for the determination of normal/abnormal blood %HbA_{1C}, were included within the linear region of the calibration curve.[18]

In summary, we have developed an efficient electroanalytical method for %HbA_{1C} assay. We employed a MP-based concentration of HbA_{1C} to create simple and amplified signaling and demonstrated the utility of a ZnMP-supported concentration method coupled to boronate-diol interactions for HbA_{1C} determination.

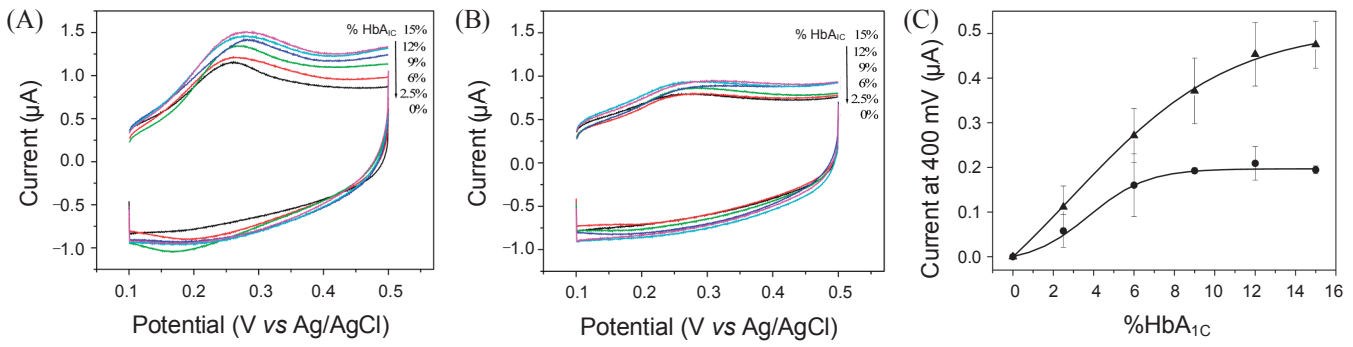


Figure 3. Electrochemical analysis of the %HbA_{1C}. Cyclic voltammograms registered for different %HbA_{1C} samples with (A) and without (B) a permanent magnet under the working electrode. (C) Calibration curves obtained for %HbA_{1C} with (triangle) and without (circle) a permanent magnet. Signals were registered from current differences at 400 mV of cyclic voltammograms. The mean of the independent triplicate analysis is shown.

Experimental Section

HbA_{1C} was purchased from Fluka and HbA_0 was from Fitzerald. FcBA, amine-terminated MP ($\Phi = 1$ μm), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), ethylene diamine tetraacetic acid (EDTA), *N*-hydroxysuccinimide (NHS), and $ZnCl_2$ were purchased from Sigma-Aldrich. To purify hemoglobin using MPs, the surface of the MPs was modified with zinc ions. The MP suspension was rinsed with a pyridine buffer (0.01 M, pH 6) and resuspended in a phosphate buffer (0.05 M, pH 8). EDTA (0.1 M) was activated in the presence of NHS (0.2 M) and EDAC (0.1 M) in a phosphate buffer at room temperature for 15 min.[15-17] Activated EDTA was added to the MPs and stirred gently at room temperature for 8 h. The EDTA-coated MPs were washed with DDW and collected 3 times with a permanent magnet. As a last step, the EDTA-coated MPs were mixed with 10 mM of zinc chloride at room temperature overnight. The final concentration of ZnMP was 50 mg/mL.

For the determination of binding capacity of ZnMPs to HbA_{1C}, various concentrations of ZnMPs were mixed with 1 mg/mL of HbA_{1C} at a ratio of 1:1 (v/v), and the solutions were set to react for 10 min at room temperature. Subsequently, the magnetic separation step was conducted, and the absorbance of the supernatants was measured at 415 nm.

For the analysis of HbA_{1C}, samples with different percentile concentrations of HbA_{1C} (0% ~ 15%) were prepared by diluting the HbA_{1C} reagent with HbA_0 in PBS. The total concentration of hemoglobin ($HbA_{1C} + HbA_0$) in the samples was adjusted to 50 μg/mL. FcBA (0.32 mM) was mixed with $\%HbA_{1C}$ samples at a ratio of 1:1 (v/v) for 20 min at room temperature. After the conjugation reaction was induced, the resulting mixture was purified using ZnMPs. The modified ZnMPs (5 mg/mL) were combined with the HbA_{1C}-FcBA conjugate for 10 min. Subsequently, ZnMPs, capturing HbA_{1C}-FcBA conjugates, were separated using a permanent magnet and washed. For comparison with a method not involving MPs, 5 mM of zinc chloride was also blended with the HbA_{1C}-FcBA conjugate at a ratio of 1:1 (v/v), according to the procedure developed in our previous work.[14]

For electrochemical signal registration, $\%HbA_{1C}$ samples were reacted under the same conditions for the HbA_{1C}-FcBA conjugation. Then, the purified product of ZnMP/HbA_{1C}-FcBA was electrochemically analyzed on a gold electrode. The evaporated gold surfaces were prepared by the resistive evaporation of 200 nm of Au onto 20 nm titanium-primed Si[100] wafers. The electrode was treated with a piranha solution containing H_2SO_4/H_2O_2 (4:1, v/v) for 5 minutes. The area of the working electrode was 0.28 cm^2, which was enough to deposit MPs as a sub-monolayer by using magnetic force. A permanent magnet was placed below the gold electrode to enhance the electrochemical signal by concentrating the conjugate at the electrolyte/electrode interface. Electrochemical measurements were carried out with an electrochemical analyzer (model 630B, CH Instruments) connected to a laptop. We used a standard three-electrode configuration with an evaporated gold working electrode, a platinum auxiliary electrode, and an external Ag/AgCl (3 M NaCl, BAS) reference electrode. Signal current levels were registered at +400 mV *vs*. Ag/AgCl from the background-subtracted cyclic voltammograms under a potential sweep rate of 5 mV/s. The electrochemical signaling cell with the magnet was separated from the potentiostat system to eliminate the influence of magnetism.

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