Biosensors and Bioelectronics 35 (2012) 355–362

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Bioelectrocatalytic detection of glycated hemoglobin (HbA_{1c}) based on the competitive binding of target and signaling glycoproteins to a boronate-modified surface

Seung Yeon Song[a], Yong Duk Han[b], Yoo Min Park[b], Chi Yong Jeong[b], Yong Ju Yang[a], Moo Sub Kim[a], Yunhee Ku[a], Hyun C. Yoon[b,*]

[a] Medical Diagnostics Team, Materials & Components Laboratory, LG Electronics Advanced Research Institute, Seoul 137724, Republic of Korea
[b] Department of Molecular Science & Technology, Ajou University, Suwon 443749, Republic of Korea

ARTICLE INFO

Article history:
Received 9 January 2012
Received in revised form 6 March 2012
Accepted 12 March 2012
Available online 17 March 2012

Keywords:
Bioelectrocatalysis
Glycated hemoglobin
HbA_{1c}
Glucose oxidase
Competitive binding

ABSTRACT

We developed an electrochemical glycated hemoglobin (HbA_{1c}) biosensor for diagnosing diabetes in whole human blood based on the competitive binding reaction of glycated proteins. Until now, no studies have reported a simple and accurate electrochemical biosensor for the quantification of HbA_{1c} in whole blood. This is because it is very difficult to correctly distinguish HbA_{1c} from large amounts of hemoglobin and other components in whole blood. To detect glycated hemoglobin, we used electrodes modified with boronic acid, which forms a covalent bond between its diol group and the cis–diol group of the carbohydrate moiety of glycated proteins. For accurate HbA_{1c} biosensing, we first removed blood components (except for hemoglobin) such as glycated proteins and blood glucose as they interfere with the boronate-based HbA_{1c} competition analysis by reacting with the boronate-modified surface via a cis–diol interaction. After hemoglobin separation, target HbA_{1c} and GOx at a predetermined concentration were reacted through a competition onto the boronate-modified electrode, allowing HbA_{1c} to be detected linearly within a range of 4.5–15% of the separated hemoglobin sample (HbA_{1c}/total hemoglobin). This range covers the required clinical reference range of diabetes mellitus. Hence, the proposed method can be used for measuring $\%HbA_{1c}$ in whole human blood, and can also be applied to measuring the concentration of various glycated proteins that contain peripheral sugar groups.

1. Introduction

The American Diabetes Association (ADA) has suggested that HbA_{1c}, the measurement of which has become a common technique for the diagnosis of diabetes mellitus, is an important factor in healthcare. As a result, they added HbA_{1c} to the diabetes diagnosis criteria in 2010. The United Kingdom Prospective Diabetes Study (UKPDS) and Diabetes Control and Complications Trials (DCCT) showed a relationship between HbA_{1c} concentration and the risk for diabetes complications (Sacks et al., 2002).

HbA_{1c} is the main form of glycated hemoglobin. It is generated from the Amadori rearrangement reaction of glucose with the *N*-terminal valine of one or both hemoglobin beta-chains (Park et al., 2008). The rate of HbA_{1c} formation is proportional to the increase in the blood glucose concentration to which erythrocytes are exposed. The level of glycation, therefore, reflects the mean concentration of blood glucose over the lifetime of the red blood cells (~120 days), and consequently, it can serve as a measure of a patient's extended metabolic control over approximately two months (Goldstein et al., 2004). HbA_{1c} is measured as the percentage of glycated hemoglobin comprising the total hemoglobin. The clinical reference range is 5–15% HbA_{1c}. The ADA has recommended that the HbA_{1c} value be maintained below than 6.5%, and suggested that specific control is needed over 6.5%. Each 1% increase in HbA_{1c} concentration corresponds to a 2 mM increase in mean blood glucose concentration (Thevarajah et al., 2009).

Quantification of HbA_{1c} has been accomplished using a number of techniques including immunoassay (Stöllner et al., 2002; Tanaka et al., 2007), ion-exchange chromatography (Lafferty et al., 2002), boronate affinity chromatography (Frantzen et al., 1995; Li et al., 2002), piezoelectric methods (Halámek et al., 2007; Přibyl and Skládal, 2006), isoelectric focusing (Mullins and Austin, 1986), colorimetry (Adamczyk et al., 2006), mass spectroscopy (Roberts et al., 2001), and electrochemical methods (Liu et al., 2006; Song and Yoon, 2009). However, these techniques have some limitations due to the complicated analytical procedures, requirements for detection labels and pre-treatment of proteins involved in signaling reactions.

* Corresponding author. Tel.: +82 312192512; fax: +82 312192394.
E-mail address: hcyoon@ajou.ac.kr (H.C. Yoon).

0956-5663/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2012.03.017

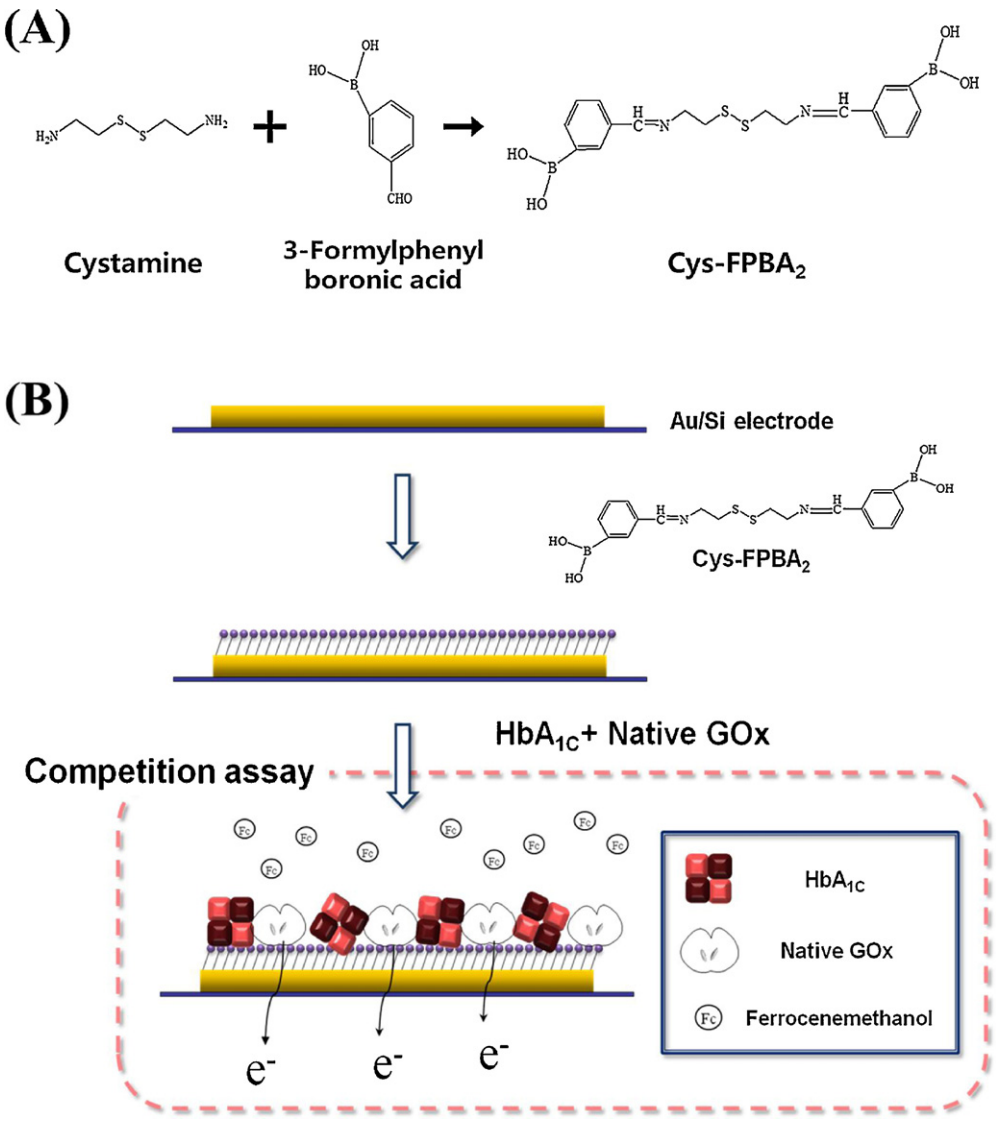

Fig. 1. (A) Synthesis of Cys-FPBA$_2$. (B) Schematic illustration of the competition assay between HbA$_{1c}$ and native GOx.

In this paper, we introduced an electrochemical biosensor for the detection of HbA$_{1c}$ in whole blood. This biosensor was constructed using the cis–diol interaction between the HbA$_{1c}$ and a boronate recognition group. Boronate covalently binds to the diol group from glycated proteins via a cis–diol interaction under alkaline conditions (Kanayama and Kitano, 2000). We newly synthesized a chemical conjugate of Cys-FPBA$_2$ and formed a boronate-modified self-assembled monolayer (SAM) as a biosensing surface (Fig. 1(A)).

To analyze blood samples, hemoglobin should be separated from blood sugars and glycated serum proteins. This is because sugars and glycated proteins in human blood will react with the boronate surface via undesired cis–diol interaction and consequently interfere with the boronate-based HbA$_{1c}$ competition analysis. To remove these interferents from the whole blood, we designed a hemoglobin separation method based on Zn ion-induced hemoglobin precipitation (vide infra, Section 2.5). Fig. 1(B) shows a separated hemoglobin sample containing HbA$_{1c}$ that was assayed by competitive binding of HbA$_{1c}$ and GOx, both glycoproteins, on the boronate-modified surface. The developed separation and signaling methods allow accurate and practical HbA$_{1c}$ diagnosis without undesired signal interference from blood sugar and glycated proteins. Here, we describe the use of this method to quantify HbA$_{1c}$ in human blood samples.

2. Experimental

2.1. Chemicals and apparatus

Cystamine, 3-formylphenylboronic acid, $Fe(CN)_6^{3-/4-}$, and ferrocenemethanol were purchased from Aldrich. GOx (from *Aspergillus niger*), β-D-glucose, and zinc chloride ($ZnCl_2$) were acquired from Sigma. Fingerprick blood samples were obtained using a lancing device from healthy volunteers who had given informed consent. HbA$_{1c}$ was purchased from Fluka, and non-glycated hemoglobin (HbA$_0$) was purchased from Fitzerald. A Lyphocheck® HbA$_{1c}$ Linearity Set was purchased from BioRad. Vivid™170 nitrocellulose membranes with 0.2 μm micropores were obtained from Pall Life Sciences. Phosphate-buffered saline solution containing 0.1 M sodium phosphate and 0.15 M NaCl (PBS, pH 7.2) and the hemoglobin separation buffer solution (HSBS, pH 7.2) containing 0.2 M HEPES, 0.05 M glycine, 0.04 M NaCl and 0.03 M $MgCl_2$ were prepared in doubly distilled and deionized water with a specific resistance greater than 18 MΩ cm. All other materials and

solvents used were of the highest quality available and purchased from regular sources.

The ^{1}H NMR spectra were recorded on a Varian Mercury Plus (400 MHz). Electrochemical measurements were carried out with an electrochemical analyzer 630B (CHInstruments). We used a standard three-electrode configuration with an evaporated gold working, a platinum auxiliary, and a Ag/AgCl reference electrode. As a reference diagnostic device, a Reflotron® Plus was purchased from Roche Diagnostics. A NycoCard® HbA_{1c} (Axis-Shield PoC) was employed to evaluate the inherent HbA_{1c} concentration of the collected whole blood.

2.2. Synthesis of Cys-$FPBA_2$

A conjugate of cystamine and 3-formylphenylboronic acid was synthesized and self-assembled on a gold surface in order to bind HbA_{1c} and GOx competitively through cis–diol interaction. Cystamine (5 mM) and FPBA (20 mM) were dissolved in DMSO and allowed to react for 4 h at 60 °C with stirring in order to obtain the Cys-$FPBA_2$ conjugate (Fig. 1(A)). Triethylamine was injected into the solution prior to creating a basic environment. The amine group of cystamine was conjugated with the aldehyde group of FPBA via Schiff's base. Conversion of the terminal amine group of cystamine into an imine group was confirmed by the shift of the ^{1}H NMR peak at δ3.10 (—CH_2—NH_2— at the terminal site of cystamine) to δ3.85 (—CH_2—N═CH— at the conjugation site of cystamine and FPBA).

2.3. Electrode fabrication

The boronate-modified gold electrode was formed as follows. Freshly evaporated gold surfaces were prepared by the resistive evaporation of 200 nm of Au onto titanium-primed (20 nm) Si-wafers, and were used as base substrates for the fabrication of sensing surfaces. Prior to the bottom-up layer formation process, the evaporated gold surfaces were cleaned by immersing them in piranha solution for 5 min. A boronate-modified SAM was formed by dipping the surfaces into a Cys-$FPBA_2$ solution in DMSO for 2 h. Next, the electrodes were rinsed with DMSO, ethanol, and DDW successively.

2.4. Confirmation of boronate SAM formation on Au electrode

The functionalization of boronate on the electrode was confirmed using cyclic voltammetry. The first test involved the use of $Fe(CN)_6^{3-/4-}$, a negatively charged electron-transferring mediator. Cyclic voltammetric measurements were first conducted in PBS at pH 7 and pH 9 in the presence of 0.1 mM $Fe(CN)_6^{3-/4-}$ under a potential sweep rate of 50 mV/s. After confirmation of the SAM formation by $Fe(CN)_6^{3-/4-}$, the second test involved the immobilization of native GOx onto the Cys-$FPBA_2$ electrode. Native GOx (25 μg/ml) in PBS (0.1 M, pH 7.2) was reacted with the boronate-modified electrode for 10 min. After rinsing with PBS, electrochemical signaling was conducted to register the current from the GOx bioelectrocatalysis. Voltammetric responses were measured in 0.1 mM ferrocenemethanol and 10 mM glucose as an enzyme substrate. All experiments included comparisons with a cystamine-modified electrode that was not conjugated with a boronate group as a control.

2.5. Application to human whole blood

2.5.1. Pretreatment of whole blood for separation of hemoglobin

Measuring %HbA_{1c} in human blood samples is essential for a clinical diagnosis of diabetes mellitus. To detect HbA_{1c} correctly, blood sugars and glycated proteins (except hemoglobin) must first be removed from the whole blood. For this purpose, a hemolysis solution (HLS) and a hemoglobin precipitation solution (HPS) were prepared. HLS (HSBS with 0.2% (w/v) Triton X-100) was used for hemolysis and for washing the remaining protein from the separation platform. HPS was prepared by dissolving $ZnCl_2$ (9 mM) in HSBS. These solutions were stored in the dark at 4 °C until use. Fig. 2(A) schematically illustrates preparation of the hemoglobin-precipitated hemolysate using Zn-ions.

A circular hemoglobin separation platform (diameter, 35 mm) was prepared using a polyester-backed nitrocellulose membrane. HLS (60 μl) was applied to the circular nitrocellulose membrane and dried at 45 °C for 1 h. The resulting HLS-treated membrane was installed on a homemade spinning machine. Fig. 2(B) shows the separation procedure. To separate hemoglobin from the whole blood, blood samples were hemolyzed by mixing 20 μl of fingerprick capillary blood with 180 μl of HLS for 5 min at room temperature. After hemolysis, 100 μl of the lysed blood samples were mixed with 100 μl of HPS for 15 min at room temperature. The zinc-induced hemoglobin precipitate mixture (8 μl) was dropped onto the HLS-treated nitrocellulose membrane on the spinning machine. Subsequently, the spinning machine was operated at 7200 rpm to generate centrifugal force on the installed membrane. During membrane spinning, 300 μl of washing solution was applied to the membrane three times to remove the potential interferents from the blood sample. After this procedure, the collected hemoglobin precipitates were resuspended in 400 μl of PBS.

2.5.2. Confirmation of separation capacity

We confirmed that the hemoglobin had been separated from the whole blood using the Bradford assay and a colorimetric glucose assay. To ensure correct determination, the separated hemoglobin was compared to the purified HbA_{1c} reagent and hemolysate (a lysed blood sample that did not undergo pretreatment for removing interfering glucose and serum proteins). First, to confirm the removal of serum proteins, spectrometric measurements of the samples were conducted and spectral data were collected. The concentration of total hemoglobin in each sample was diluted to 100 μg/ml for spectrometry and spectra were collected over the range of 270–600 nm wavelength. The Bradford assay was then performed for quantification of the remaining serum proteins in each sample.

For the enzyme-based colorimetric glucose assay, 50 μl of enzyme–chromogen mixture was loaded onto a 96-well plate. Then, 50 μl of each test sample (non-treated hemolysate and collected hemoglobin samples that underwent 0–3 washing steps) was injected into the enzyme–chromogen mixtures and incubated for 20 min at 37 °C. A 300 μl of washing solution was added per washing step. Finally, the absorbance at 630 nm was measured for each collected sample. A standard calibration curve for glucose concentration was generated by the same assay using different glucose concentrations (0–80 μg/ml) for comparison.

2.6. Electrochemical % HbA_{1c} determination with HbA_{1c}–GOx competition assay

To prepare various concentrations of HbA_{1c} samples, total hemoglobin concentration and %HbA_{1c} of collected whole blood was first measured using Reflotron® Plus and Nycocard® before spiking with HbA_{1c} (the %HbA_{1c} of the donated blood was 4.5%). Next, hemoglobin was separated from the whole blood were spiked with HbA_{1c} at 6%, 7%, 9%, 12%, and 15% concentrations. For the competition assay, the prepared HbA_{1c} samples were diluted to 2 μg/ml and mixed with a fixed concentration of GOx (total 20 μg/ml) in PBS, and this mixture was applied to the Cys-$FPBA_2$ electrode for 10 min. After washing with PBS, electrochemical signaling was conducted to register the current from the GOx bioelectrocatalysis. Voltammetric measurements were conducted in PBS in the

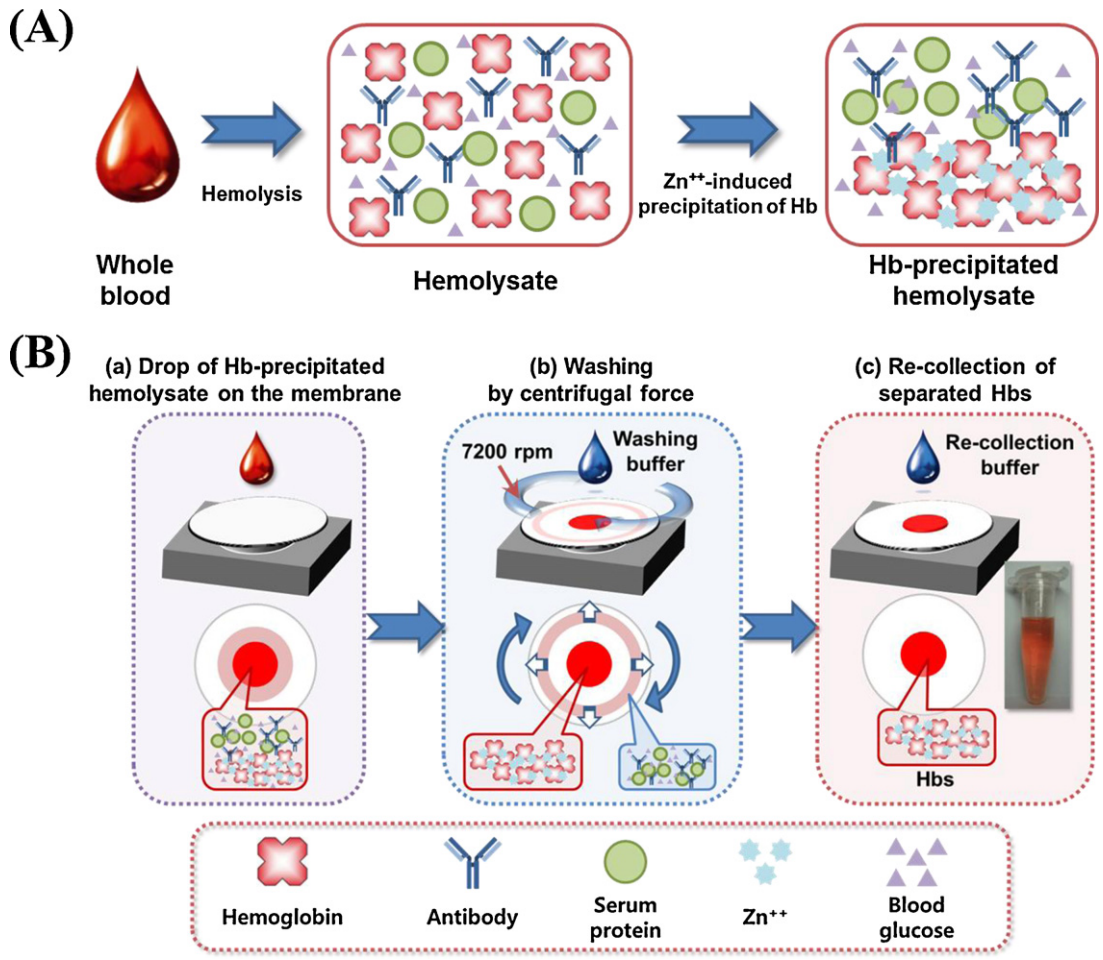

Fig. 2. (A) Schematic illustration of the hemolysis and zinc-induced precipitation. (B) Hemoglobin separation process using zinc-induced hemoglobin precipitation and centrifugal force.

presence of 0.1 mM ferrocenemethanol and 10 mM glucose under 5 mV/s scan rate.

3. Results and discussion

3.1. Confirmation of boronate SAM formation on Au by cyclic voltammetry

Boronate is known to assume an anionic form in alkaline solutions due to the addition of an OH^- ion to the boron atom (Takahashi and Anzai, 2005). This property was used to confirm the presence of a functionalized boronate group on the electrode surface. In this experiment, PBS solutions of pH 9 and pH 7, respectively, were chosen for the alkaline and control (neutral) conditions. In addition, we used $Fe(CN)_6^{3-/4-}$ as a negatively charged electron-transferring mediator.

Fig. 3 shows cyclic voltammograms for the $Fe(CN)_6^{3-/4-}$ ion on the Cys-FPBA$_2$ and cystamine electrode surfaces for each condition. In Fig. 3(A)-left, the cyclic voltammogram of the cystamine electrode at pH 7 is similar to that at pH 9. The peak currents at pH 7 and pH 9 are 2.5 and 2.6 μA, respectively, at 230 mV vs. Ag/AgCl reference electrode. However, the voltammograms of the Cys-FPBA$_2$ electrode differed significantly at pH 7 and pH 9 (Fig. 3(A)-right). First, the cyclic voltammogram of the Cys-FPBA$_2$ electrode showed no distinguishable redox peak at pH 9. Currents sampled at 230 mV decreased from 3.3 μA to 1.78 μA when the pH was altered from 7 to 9 (Fig. 3(A)-right). This is due to the electrostatic repulsion between the $Fe(CN)_6^{3-/4-}$ ion and the negatively charged surface of the boronate-modified electrode under alkaline conditions. Electrostatic repulsion interferes with the electron-transferring reaction of $Fe(CN)_6^{3-/4-}$. The voltammetric response of the Cys-FPBA$_2$ electrode confirmed that the boronate group was successfully functionalized on the sensor surface.

In addition, to confirm the cis–diol interaction between glycoproteins and boronate groups on the electrode surface, native GOx was reacted with the Cys-FPBA$_2$ and the cystamine electrodes. GOx was employed for the signal generation because it is a well-known electrocatalyzable enzyme as well as a glycated protein. GOx contains 16% neutral sugars and 2% amino sugars.

Fig. 3(B) shows schematic illustrations of the reactions at the sensor surface and the bioelectrocatalytic signal from GOx in the presence of 0.1 mM ferrocenemethanol as an electron-transferring mediator and 10 mM glucose as an enzyme substrate. The voltammogram of the cystamine electrode obtained (Fig. 3(B)-left) in the presence of 10 mM glucose was similar to that acquired in the absence of glucose. As the cystamine electrode had only an amine group, it had not reacted with GOx. The small increment in anodic current from the nonspecific binding of GOx on the cystamine surface (Fig. 3(B)-left) is discussed in more detail in Supplementary information. The native GOx molecules cannot be immobilized on the electrode because the amine group is not a useful group for GOx immobilization. Therefore, bioelectrocatalysis of GOx and signal amplification did not occur in the cystamine electrode.

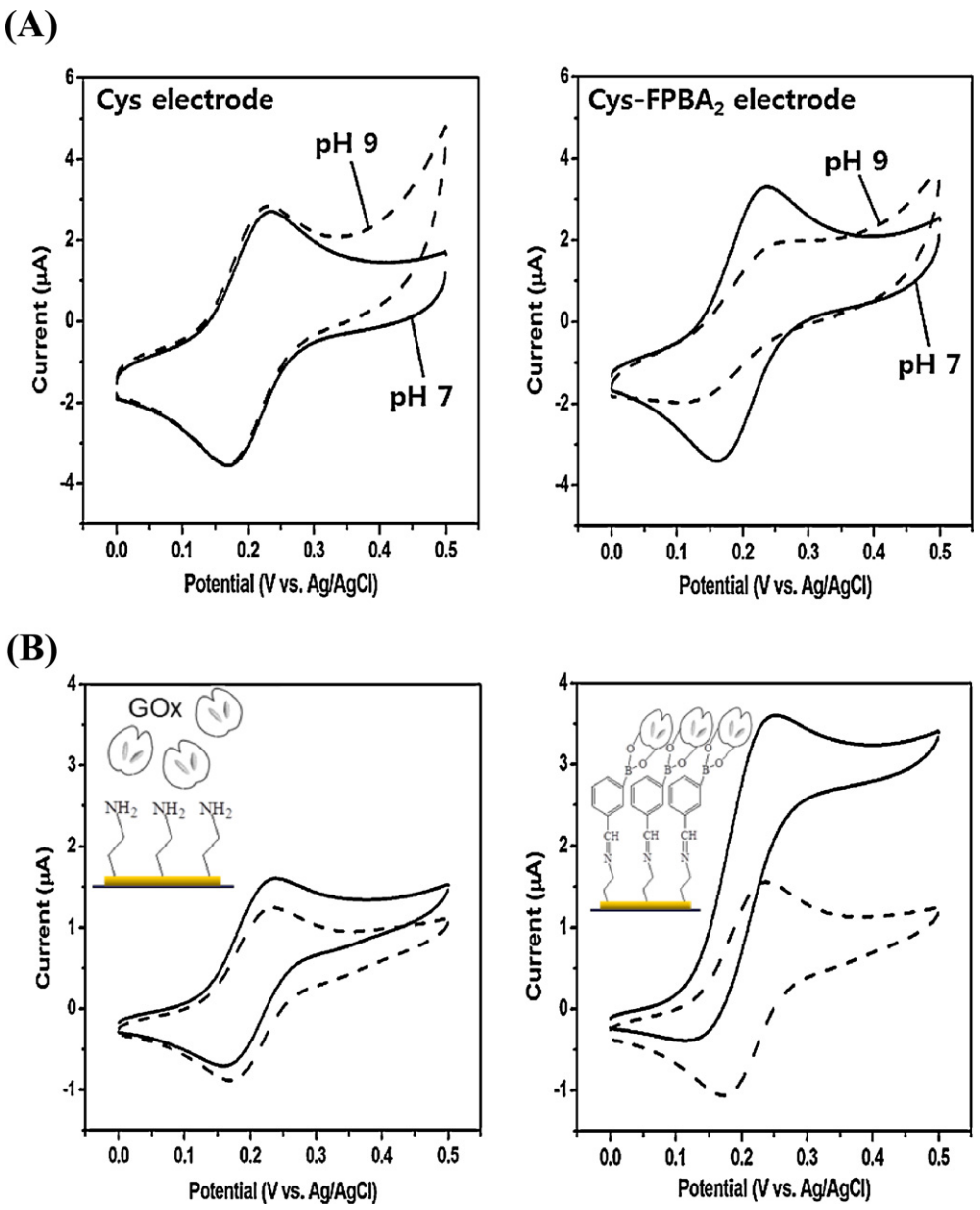

Fig. 3. (A) Cyclic voltammograms for the 0.1 mM $Fe(CN)_6^{3-/4-}$ on the cystamine (A-left) and Cys-$FPBA_2$ (A-right) electrodes under neutral (pH 7, solid lines) and alkaline (pH 9, dashed) conditions. Measurements were performed under a potential sweep rate of 50 mV/s. (B) Cyclic voltammograms of native GOx binding on the cystamine (B-left) or Cys-$FPBA_2$ electrodes (B-right). Cyclic voltammograms show signal amplification by bioelectrocatalysis due to the immobilized GOx. Measurements were performed in the presence of 0.1 mM ferrocenemethanol with 10 mM glucose in a 0.1 M PBS (pH 7.2) under a potential sweep rate of 5 mV/s. Voltammetric signal traces were registered before (dashed) and after (solid) the addition of glucose.

For the immobilization of GOx on the amine-functionalized cystamine electrode, the enzyme generally requires pretreatment with a solution such as sodium periodate, which oxidizes peripheral carbohydrates.

In comparison, the voltammogram of the Cys-$FPBA_2$ electrode was different from that of the cystamine electrode in the presence of 10 mM glucose. Fig. 3(B)-right shows a current amplified by enzymatic catalysis of GOx on the Cys-$FPBA_2$ electrode. The current at the Cys-$FPBA_2$ electrode increased by about 2.2 μA at 400 mV vs. Ag/AgCl due to the bioelectrocatalytic reaction of surface-bound native GOx molecules. Unlike the cystamine electrode, native GOx could be immobilized on the Cys-$FPBA_2$ electrode via cis–diol interactions with no pretreatment. From these results, we conclude that the Cys-$FPBA_2$ electrode is useful in that it specifically binds glycated proteins including GOx and target HbA_{1c}.

3.2. Evaluation of separated hemoglobin

For accurate HbA_{1c} quantification, it is important to remove the components that contain carbohydrates from the whole blood because they interfere with the reaction between HbA_{1c}/GOx and the boronic acid group. In this study, hemoglobin separation was accomplished using zinc-induced hemoglobin precipitation and washing out unwanted blood components using centrifugal force. In previous studies, Frantzen (Frantzen et al., 1997) demonstrated that adding zinc cations to hemolysates induces selective hemoglobin precipitation due to the specific interaction between zinc cations and hemoglobin. They also showed that the hemoglobin/zinc ratio can be used to determine the amount and specificity of precipitation. Thus, an increase in the concentration of zinc ions accelerates the precipitation of the hemolysate as well as

the co-precipitation of plasma proteins. In our study, hemoglobin present in the hemolysate precipitated rapidly as the concentration of $ZnCl_2$ was increased linearly from 3 to 18 mM. Based on these results, the optimum concentration for the precipitation of hemolysates was determined to be 9 mM $ZnCl_2$. This is the minimum concentration required to begin precipitation and to maintain hemoglobin in a stable form.

After hemolysis, the samples were placed on a nitrocellulose membrane and components of the precipitated hemolysates were separated horizontally due to differences in size and density. To enhance the efficiency of the horizontal separation of the precipitated hemoglobin and other blood components, we introduced centrifugal force during the washing step. Most of the precipitated hemoglobin was retained on the membrane during the washing out step. All components except the precipitated hemoglobin (for instance, glucose and unprecipitated proteins) were washed out from the inside to the outside during the washing step due to their relatively small size. Hemoglobin was collected in the center of the membrane in the form of a semi-gel. To prepare a sample of soluble hemoglobin, the precipitated hemoglobin was redissolved in PBS. This experimental procedure is schematically summarized in Fig. 2(B). To confirm the separation of hemoglobin from whole blood, we performed spectroscopic measurements, a Bradford assay and an enzyme-based colorimetric assay.

Fig. 4(A) shows the absorption spectra of the hemolysates (black line), separated hemoglobin (red), and purified HbA_{1c} reagent (blue) from 270 to 600 nm. Hemoglobin has inherent absorbance peaks at 415 nm and at 525–600 nm. To verify the separation of hemoglobin, samples were evaluated by measuring the absorbance at 415 nm for hemoglobin and at 280 nm for total proteins. As shown in Fig. 4(A), the amount of hemoglobin is almost identical since the peak at 415 nm is nearly equal. In contrast to the absorbance at 415 nm, the peak height at 280 nm is different, indicating a change in the amount of total protein. The absorbance at 280 nm for the separated hemoglobin decreased after pretreatment of the hemolysate using the spinning machine. Moreover, the spectrum of the separated hemoglobin is similar to the spectrum of purified HbA_{1c} reagent. These results indicate that other serum proteins except hemoglobin have been removed from the hemolysate. The purity of the separated hemoglobin was nearly equal to that of the purified HbA_{1c} reagent.

To evaluate the purity of the separated hemoglobin, the amount of total protein in the samples was quantitatively measured using the Bradford assay with bovine serum albumin as the standard. Fig. 4(B) shows the total protein concentrations in the hemolysate, separated hemoglobin and purified HbA_{1c} reagent. The amount of total protein in the separated hemoglobin was found to be 16 μg/ml less than that in the hemolysate, and it was also approximately 4 μg/ml higher than that of the purified HbA_{1c} reagent. The latter difference seems to be due to the limited performance of the spinning machine.

Fig. 4(C) shows the changes in blood glucose concentrations in each sample. Even though the hemoglobin concentrations in the samples were unchanged, their glucose concentrations were decreased significantly as a result of the washing steps in the blood pretreatment process. The glucose concentration of the 40-fold diluted hemolysate as a negative control was 19.3 μg/ml (Fig. 4(C)). As the number of washing steps increased from zero to three, the glucose concentration decreased gradually from 2.9 μg/ml to 0.7 μg/ml, respectively. These results show that the glucose concentration of the thrice washed sample decreased about 96.4% compared with the glucose concentration of the negative control. Based on these results, we can conclude that the hemoglobin was successfully separated from whole blood by the proposed hemoglobin separation process.

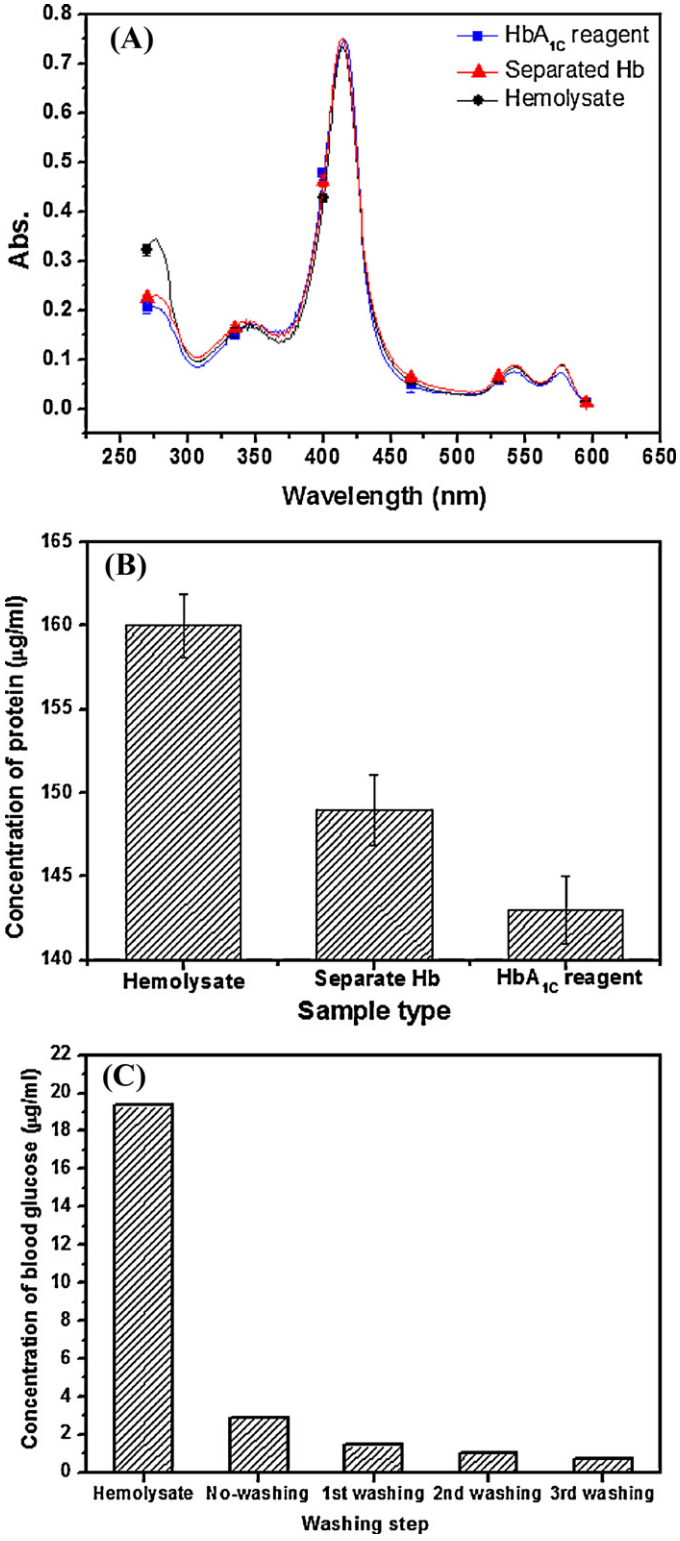

Fig. 4. Confirmation of the removal of potential interferents. (A) Absorption spectra changes during the pretreatment process. The peaks at 280 nm and 425 nm indicate the alteration of total protein and hemoglobin content. The spectra show similarities and differences in peak height between the hemolysate (black), separated hemoglobin (red), and purified HbA_{1c} reagent samples (blue). (B) Quantitative measurement (triplicate) of total protein in each sample using the Bradford assay. (C) Results of the colorimetric glucose assays with samples that underwent a varying number (0–3) of washing steps. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

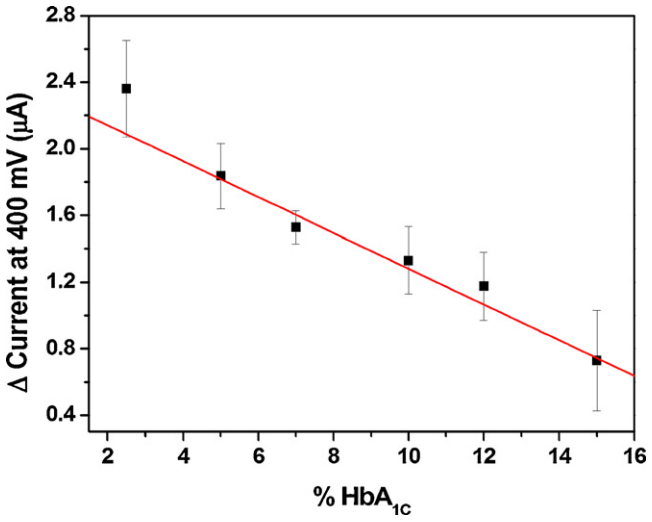

Fig. 5. Quantification of HbA_{1c} concentration based on a competition assay in the purified HbA_{1c} samples. Each data point represents the average and standard deviation of independent triplicate tests.

3.3. Electrochemical determination of the %HbA_{1c} with an HbA_{1c}/GOx competition assay

We detected HbA_{1c} quantitatively via an HbA_{1c}/GOx competitive reaction. To detect HbA_{1c} binding on the Cys-$FPBA_2$ electrode, we used GOx to amplify the electrochemical signal by enzymatic catalysis. Prior to the real blood sample assay, the HbA_{1c}/GOx competition assay was performed to assess the effectiveness of this system for use as an HbA_{1c} biosensor using purified 'HbA_{1c} + HbA_0' reagent. To prepare HbA_{1c} reagent samples of various concentrations, different concentrations of HbA_{1c} (2.5–15%) were serially diluted with HbA_0. Total hemoglobin concentrations in all samples were adjusted to the normal level of an adult human (150 mg Hb/ml). Then, the HbA_{1c}/HbA_0 samples were diluted to 2 μg/ml and assayed.

Fig. 5 shows the results of the competition assay for the purified 'HbA_{1c} + HbA_0' sample. Anodically amplified currents were observed at +400 mV vs. Ag/AgCl in the respective cyclic voltammograms. Signal current levels were determined from the background-subtracted voltammograms for respective analyte concentrations. The signal decreased proportionally with an increase in %HbA_{1c} from 2.5% to 15%. The obtained calibration result fell within the clinical reference range, which indicates that our system could serve as a diagnostic tool in diabetes control. Therefore, our Cys-$FPBA_2$ SAM is useful as an electrochemical HbA_{1c} biosensing interface.

Demonstrating the ability of the proposed diagnostic system to detect HbA_{1c} in real human whole blood is essential before it can be implemented clinically. Whole human blood contains not only hemoglobin, but also certain concentrations of glucose and other glycated proteins. Because boronic acid can bind these sugars, blood glucose interferes with the conjugation between HbA_{1c} and boronic acid. To correctly quantify the HbA_{1c} in whole blood, we employed our hemoglobin separation process followed by the competition assay. To evaluate the necessity of hemoglobin separation and the potential for the developed HbA_{1c} detection method using the spinning machine to be applied in clinical diagnosis using human samples, the hemolysate and the HbA_{1c} linearity set from BioRad were also assayed. In the case of HbA_{1c} linearity set, to compare the results accurately, the HbA_{1c} concentrations of control samples were first determined by VARIANT™ II TURBO HbA_{1c} Kit and then applied to the electrochemical HbA_{1c}–GOx competition assay.

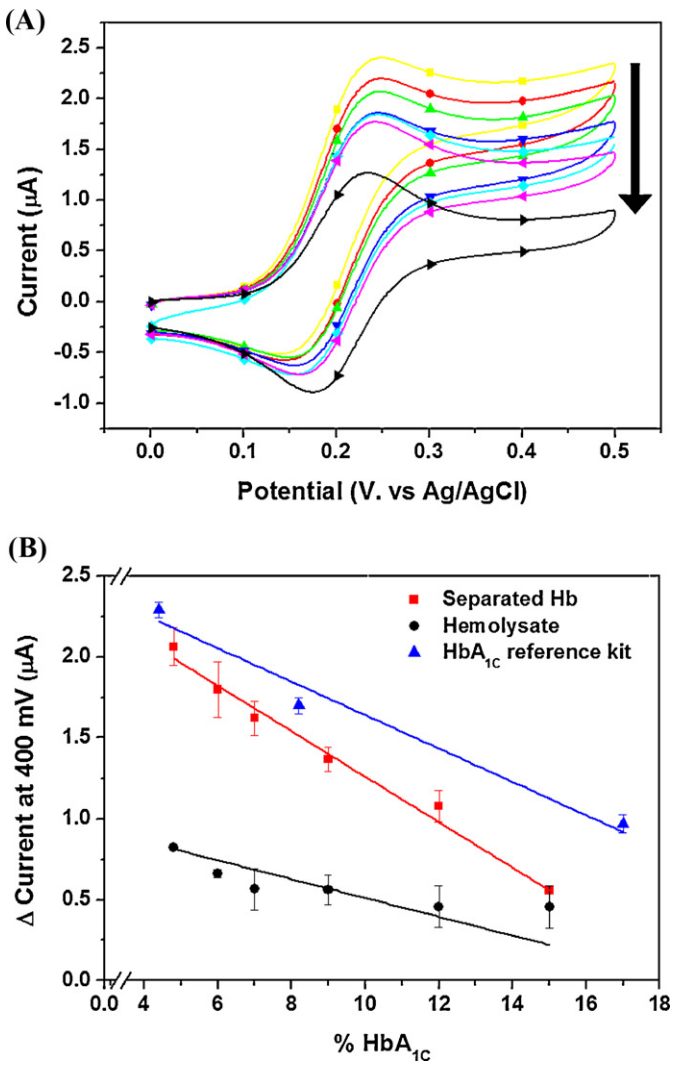

Fig. 6. Electrochemical HbA_{1c} sensing. (A) Cyclic voltammograms of the competition assay between HbA_{1c} and native GOx. Measurements were performed in the presence of ferrocenemethanol (0.1 mM) and glucose (10 mM) under 5 mV/s. (B) Calibration curves as a function of %HbA_{1c} in each sample type (hemolysate, separated hemoglobin, and HbA_{1c} reference kit). The mean of independent triplicate analyses is shown, and error bars indicate standard deviations. Signals were registered at +400 mV vs. a Ag/AgCl electrode from the background-subtracted voltammograms.

Fig. 6 shows the calibration curve obtained from each hemoglobin sample/GOx competition assay. The signal current of the hemolysate was indistinguishable from the concentration gradient of %HbA_{1c} (black line). However, the signal current of the separated hemoglobin (red) and HbA_{1c} reference sample (blue) decreased as the %HbA_{1c} concentration increased because of the interference of HbA_{1c} with GOx binding to the Cys-$FPBA_2$/Au surface. The red and blue lines in Fig. 6 show that the detection range of the HbA_{1c} reference sample and the separated hemoglobin samples was 4.5–15%, covering the detection range desired for the HbA_{1c} assay. In addition, the sensitivity of our assay in the separated hemoglobin sample (0.14 μA/%HbA_{1c}, R^2 = 0.99) was very similar to that in the HbA_{1c} reference samples (0.1 μA/%HbA_{1c}, R^2 = 0.96).

However, in the hemolysate, the calibration results were not concentration dependent for relatively high concentrations of components including blood sugar, albumin, and immunoglobulin in whole blood. Blood sugars and other glycated proteins can bind to immobilized boronic acid on the electrode, and serum protein might adsorb on the electrode surface. Thus, blood components interfere with the interaction between HbA_{1c} and the

functionalized boronic acid on the electrode. Therefore, the removal of these components would be critical for accurate detection of HbA_{1c} in blood samples. In addition, the coefficients of variation of the HbA_{1c} reference kit samples and separated hemoglobin samples for this assay were found to be approximately 4% and 6%, respectively, supporting the potential of this method in diagnostic analysis. Based on these observations, we suggest that the developed blood pretreatment method and competitive binding assay of target glycoprotein on the boronate-modified surface would be useful for HbA_{1c} determination.

4. Conclusions

In this study, we developed a novel HbA_{1c} electrochemical biosensor with a boronate-modified electrode surface. Currently, electrochemical analyses of HbA_{1c} have been performed using proteins such as anti-HbA_{1c} antibody and haptoglobin. However, their clinical translation and commercialization is difficult due to the limited availability and cost of the proteins. The main advantage of the method developed in this study is its simplicity. Using the HbA_{1c}–GOx competition assay on the Cys-$FPBA_2$/Au electrode and separation system, the HbA_{1c} concentration can be easily determined without the use of other detection labels such as antibodies, dyes, or fluorescent materials. Our results suggest that the boronate-modified surface can be developed into electrochemical biosensors for glycated proteins.

Acknowledgment

This research was supported by the National Research Foundation (2011-0025819, 2011-0007109) and the Priority Research Centers Program (2011-0022978) funded by MEST, Korea.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2012.03.017.

References

Adamczyk, M., Chen, Y.Y., Johnson, D.D., Mattingly, P.G., Moore, J.A., Pan, Y., Reddy, R.E., 2006. Bioorg. Med. Chem. Lett. 16, 1324–1328.
Frantzen, F., Grimsrud, K., Heggli, D.E., Sundrehagen, E., 1995. J. Chromatogr. B 670, 37–45.
Frantzen, F., Grimsrud, K., Heggli, D.E., Sundrehagen, E., 1997. Hemoglobin 21, 155–172.
Goldstein, D.E., Nathan, D., Little, R.R., Peterson, C.M., Lorenz, R.A., Sacks, D.B., Malone, J.I., 2004. Diabetes Care 27, 1761–1773.
Halámek, J., Wollenberger, U., Stöklein, W.F.M., Scheller, F.W., 2007. Electrochim. Acta 53, 1127–1133.
Kanayama, N., Kitano, H., 2000. Langmuir 16, 577–583.
Lafferty, J.D., McFarlane, A.G., Chui, D.H.K., 2002. Arch. Pathol. Lab. Med. 126, 1494–1500.
Li, Y., Jeppsson, J.O., Karlesson, M.J., Larsson, E.L., Jungvid, H., Galaev, I.Y., Mattiasson, B., 2002. J. Chromatogr. B 776, 149–160.
Liu, S., Wollenberger, U., Katterle, M., Scheller, F., 2006. Sens. Actuators B 113, 623–629.
Mullins, R.E., Austin, G.E., 1986. Clin. Chem. 32, 1460–1463.
Park, J.Y., Chang, B.Y., Nam, H.H., Park, S.M., 2008. Anal. Chem. 80, 8035–8044.
Přibyl, J., Skládal, P., 2006. Biosens. Bioelectron. 21, 1952–1959.
Roberts, N.B., Amara, A.B., Morris, M., Green, B.N., 2001. Clin. Chem. 47, 316–321.
Sacks, D.B., Bruns, D.E., Goldstein, D.E., Maclaren, N.K., Mcdonald, J.M., Parrott, M., 2002. Clin. Chem. 48, 436–472.
Song, S.Y., Yoon, H.C., 2009. Sens. Actuators B 140, 233–239.
Stöllner, D., Stöcklein, W., Scheller, F., Warsinke, A., 2002. Anal. Chim. Acta 470, 111–119.
Takahashi, S., Anzai, J., 2005. Langmuir 21, 5102–5107.
Tanaka, T., Tsukube, S., Izawa, K., Okochi, M., Lim, T.K., Watanabe, S., Harada, M., Matsunaga, T., 2007. Biosens. Bioelectron. 22, 2051–2056.
Thevarajah, M., Nadzimah, M.N., Chew, Y.Y., 2009. Clin. Biochem. 42, 430–434.