analytical chemistry

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Determination of Percent Hemoglobin A1c Using a Potentiometric Method

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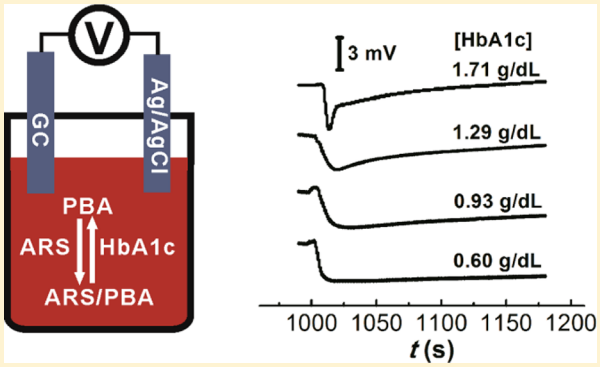
Supporting Information

ABSTRACT: We report a potentiometric method for measuring the hemoglobin A1c (HbA1c, glycated hemoglobin) concentration, hemoglobin (Hb) concentration, and percent HbA1c (%HbA1c) in human blood hemolysate. The %HbA1c is important for diagnosis and management of diabetes mellitus. Alizarin red s (ARS) is used as a redox indicator. Phenylboronic acid (PBA) binds to both ARS and HbA1c via diol–boronic acid complexation. The binding of PBA to ARS shifts its redox potential negatively. However, when HbA1c competes with ARS for PBA binding, the solution potential shifts positively. This shift is linked to the HbA1c concentration. The concentration of Hb is determined by allowing it to react with $Fe(CN)_6^{3-}$. The potential shift arising from the reduction of $Fe(CN)_6^{3-}$ by Hb is proportional to the logarithm of the Hb concentration. The results obtained for %HbA1c in human blood hemolysate are in good agreement with those determined using a reference method.



Diabetes mellitus is a growing worldwide health problem.[1] Diagnosis and management of this disease require continuous monitoring of blood glucose levels, usually using personal glucose meters (PGMs).[2] However, effective management requires three to four tests a day owing to the large fluctuation of glucose levels, and hence, this type of spot testing does not reflect the average blood glucose level over a prolonged period. For the latter type of determination, testing for percent hemoglobin A1c (glycated hemoglobin, %HbA1c), which is the ratio of HbA1c to total hemoglobin (Hb) concentration, is more appropriate.[3]

HbA1c is formed by a nonenzymatic reaction between blood glucose and the N-terminal valine of the Hb β chain, followed by an Amadori rearrangement reaction.[4] The correlation between %HbA1c and the average blood glucose level is well established.[5] Specifically, %HbA1c provides a running average of the blood glucose level over the half-life of erythrocytes (i.e., ~60 days) and has therefore been recognized as a crucial parameter for the diagnosis and management of diabetes mellitus.[5] The 2010 American Diabetes Association Standards of Medical Care in Diabetes has included %HbA1c $\geq$ 6.5% as a criterion for the diagnosis of diabetes mellitus.[6]

A wide range of methods have been developed for the determination of %HbA1c, including boronate affinity chromatography,[7,8] ion-exchange chromatography,[9] electrophoresis,[10] fluorescence,[11,12] UV–vis absorption,[12] chemiluminescence,[4] electrochemical methods,[13−17] and immunoassays.[18,19] However, most of these methods are based on chromatographic separations. Other methods usually require antibody reagents or commercially unavailable derivatives of boronic acid, which render these methods relatively vulnerable and expensive.[4,11,12,16,18,19] Moreover, some of these methods only determine the absolute concentration of HbA1c rather than %HbA1c. Finally, it has recently been reported that the majority of commercially available HbA1c analyzers are unreliable.[20]

Electrochemical methods, because of their low cost and simplicity, are particularly amenable to point-of-care sensing.[21,22] For example, we previously reported electrogenerated chemiluminescent detection of HbA1c using a microelectrochemical bipolar electrode array on a microfluidic chip.[15] As discussed earlier, however, this approach leads to the absolute concentration of HbA1c rather than the more useful %HbA1c. Here, we report a method based on potentiometry for determination of the HbA1c concentration, Hb concentration, and thus %HbA1c in human blood hemolysate. The method is simple and requires no chromatographic separation, and all necessary reagents are inexpensive, stable (not biological), and commercially available. The quantitative determination of HbA1c is based on measuring the potential shift of a redox indicator, alizarin red s (ARS, US ~$2.1/g). When ARS binds to phenylboronic acid (PBA; US ~$2.3/g) through diol–boronic acid complexation, the redox potential shifts negatively. In the presence of HbA1c, which is a competitor of ARS for PBA binding, the potential shifts back positively. The

Received: November 5, 2012
Accepted: December 6, 2012
Published: December 20, 2012

   dx.doi.org/10.1021/ac3032228 | Anal. Chem. 2013, 85, 1834–1839

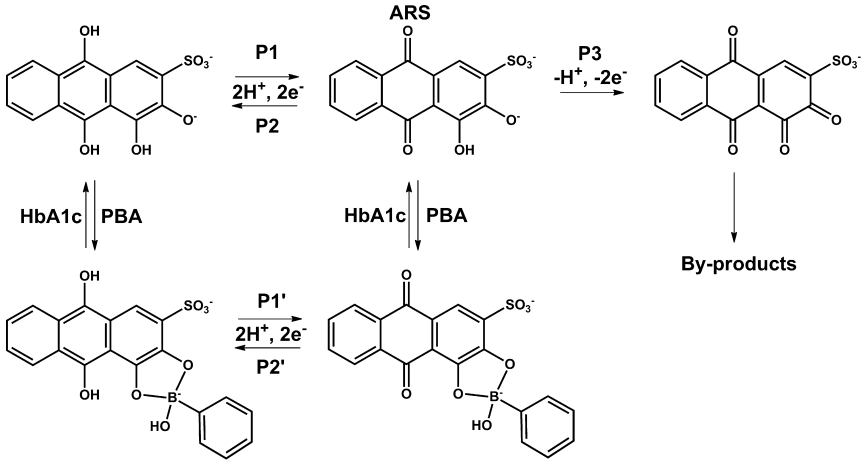


Figure 1. Reaction scheme showing the complexation and redox processes used for the HbA1c assay. P1, P2, P1′, P2′, and P3 correspond to the redox processes identified in Figure 2.

magnitude of this back shift is used for the quantification of HbA1c. For the determination of total Hb, the blood hemolysate sample is allowed to react with $K_3Fe(CN)_6$, and the potential shift owing to the reduction of $K_3Fe(CN)_6$ by Hb is correlated quantitatively with the Hb concentration.

EXPERIMENTAL SECTION

Chemicals and Materials. Lyophilized Hb standard (13.2 g/dL) and a HbA1c calibrator kit with four lyophilized Hb samples containing 5.0%, 8.05, 10.8%, and 14.8% HbA1c, respectively, were purchased from Pointe Scientific, Inc. (Canton, MI). The concentrations of total Hb in the HbA1c calibrator samples were determined using UV−vis spectroscopy by comparing the adsorption at 415 nm with that of Hb standards. The absolute concentration of HbA1c was then obtained by multiplying the total Hb concentration and %HbA1c. ARS, $K_3Fe(CN)_6$ (99+%), and *N*-ethylmorpholine (99%) were purchased from Acros Organics. PBA (≥97%) was purchased from Sigma-Aldrich. KCl and HCl were purchased from Fisher Scientific. All solutions were prepared using deionized water (18.0 MΩ·cm, Milli-Q gradient system, Millipore). All reagents were used as received.

Electrochemical Measurements. All electrochemical measurements were made using a CHI650c potentiostat from CH Instruments (Austin, TX). For cyclic voltammetry (CV) and linear sweep voltammetry (LSV), a glassy carbon working electrode (3.0 mm in diameter), a Ag/AgCl reference electrode, and a platinum counter electrode were used. For potentiometric measurements, only the glassy carbon electrode and the Ag/AgCl electrode were required. The Ag/AgCl reference electrode was filled with saturated KCl solution. Before all electrochemical measurements, the glassy carbon electrode was polished with slurries of α-alumina (300 nm and then 50 nm) using a MetaServ-3000 polisher from Buehler (Lake Bluff, IL), rinsed with deionized water, and dried under nitrogen.

Determination of the Dissociation Constant of ARS−PBA. The dissociation constant for ARS−PBA complexation was determined by nonlinear fitting of a titration curve using Origin 8.0 with the function given in the following equation:[23]

$$C_{PBA} = C_{ARS}(j - j_0)/(j_\infty - j_0) + K_d(j - j_0)/(j_\infty - j) \quad (1)$$

Here, C_{PBA} is the total concentration of PBA and C_{ARS} is the total concentration of ARS. The measured peak current density is j, which is proportional to the concentration of free ARS. The peak current density measured in the absence of PBA is j_0. The peak current measured when PBA is in excess is j_∞.

Potentiometric Detection of HbA1c. A 100 μL aliquot of the Hb sample was mixed with 50 μL of 0.10 M *N*-ethylmorpholine buffer (pH adjusted to 8.5 using HCl) containing 1.0 mM ARS and 0.10 M KCl in a glass vial with stirring (the ARS stock solution was freshly prepared each day). The solution was diluted to 500 μL using 0.10 M *N*-ethylmorpholine buffer (pH 8.5) containing 0.10 M KCl. The open-circuit potential (OCP) of the stirred solution was measured for a total of 1180 s at a sampling rate of 10 per second. At 1000 s, 1.0 μL of 2.0 M PBA dissolved in ethanol was added to the glass vial. The resulting potential shift was recorded, and the dose−response curve was plotted for the determination of the HbA1c concentration.

Potentiometric Detection of Total Hb. A 3.0 mL aliquot of 0.010 M PBS solution (pH 7.4) containing 10 mM $K_3Fe(CN)_6$ was freshly prepared and added to a glass vial with stirring. The OCP was measured at a sampling rate of 10 per second with stirring. At 60 s, 3.0 μL of Hb sample was added to the glass vial. After 200 s, an additional 3.0 μL aliquot of the Hb sample was added to the glass vial. The resulting potential shift during the second addition of sample was used for the determination of the total Hb concentration.

RESULTS AND DISCUSSION

Electrochemistry of ARS and ARS−PBA. Schumacher et al. have examined the electrochemistry of ARS and ARS−PBA using CV, and they also used ARS as an amperometric indicator for studying the binding between PBA and fructose in phosphate buffer (pH 7.4).[24] The binding between diols and boronic acid is generally believed to be more favorable at higher pH values,[25] and therefore, we used 0.10 M *N*-ethylmorpholine buffer (pH 8.5) containing 0.10 M KCl supporting electrolyte for all electrochemical experiments involving ARS. At pH 8.5, it is known that the 3-hydroxy group of ARS is deprotonated,[26] as shown in Figure 1.

A cyclic voltammogram of 1.0 mM ARS is shown in Figure 2a (black curve). A nearly reversible process is observed at $E_{1/2}$ = −704 mV with ΔE = 53 mV (P1 and P2 in Figure 2a). Recall

dx.doi.org/10.1021/ac3032228 | *Anal. Chem.* 2013, 85, 1834−1839

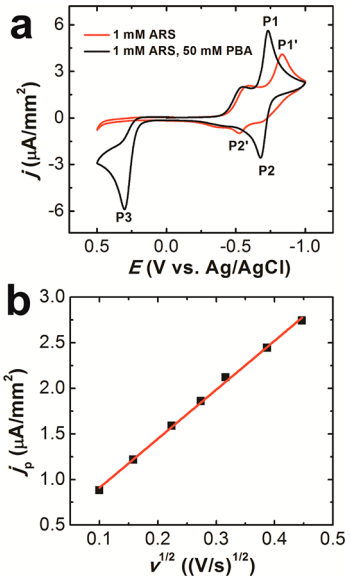


Figure 2. (a) Cyclic voltammograms obtained in solutions containing 0.10 M *N*-ethylmorpholine buffer (pH 8.5), 1.0 mM ARS, and 0.10 M KCl. A 50 mM concentration of PBA was added to this solution to obtain the red CV. Scan rate 0.100 V/s. (b) Peak current density (P3) as a function of the square root of the scan rate.

that $E_{1/2} = (E_{pc} + E_{pa})/2$ and $\Delta E = E_{pc} - E_{pa}$, where E_{pc} and E_{pa} are the potentials corresponding to the cathodic and anodic current density peaks, respectively. An irreversible process is detected at E_{pa} = 301 mV (P3 in Figure 2a). The nearly reversible and the irreversible processes are attributable to the two-proton two-electron redox reactions of the orthoquinone moiety and the oxidation of the dihydroxyl moiety of the ARS, respectively (Figure 1).[24] The peak at E_p = −592 mV corresponds to the reduction of oxygen. This assertion is confirmed by Figure S1 in the Supporting Information, which shows that this peak is absent in a deoxygenated solution. All subsequent electrochemical measurements were carried out in air-saturated solutions, because it is difficult to deoxygenate Hb solutions.

When 50 mM PBA is added to the 1.0 mM ARS solution, the voltammetry changes (red curve, Figure 2a). In this case, peak P3 is not present due to complexation between ARS and PBA via the diol−boronic acid interaction (Figure 1). Furthermore, the peak potentials of the orthoquinone redox process (P1′ and P2′ in Figure 2a) shift, they become more irreversible (ΔE = 310 mV and $E_{1/2}$ = −679 mV), and the oxygen reduction reaction shifts slightly negatively (E_p = −592 mV).

As shown in Figure 2b, the peak current density arising from the dihydroxyl oxidation of ARS (peak P3) is proportional to the square root of the scan rate (10−200 mV/s), indicating that the redox reaction is diffusion limited.[27] Therefore, the magnitude of the P3 peak current density is proportional to the bulk concentration of free ARS in the solution, and hence, it can be used to study the binding between ARS and PBA.

Complexation of ARS and PBA. A detailed fluorescence study of the complexation between PBA and a range of diols, including ARS, was reported by Wang et al.[25] One outcome of this study is that the pH and solution composition greatly influence the binding of PBA and diols.[25] Generally, the interactions are favored at high pH values when the boronic acid is tetrahedral and negatively charged, as shown in Figure 1.[25]

To determine the time required for the ARS−PBA complexation reaction to take place under electrochemical conditions, 10 μL of 1.0 M PBA dissolved in ethanol was added to 10 mL of 0.10 M *N*-ethylmorpholine buffer containing 1.0 mM ARS and 0.10 M KCl. Linear sweep voltammograms were obtained as a function of time, and the resulting peak current densities, j_p, as a function of time are presented in Figure 3a.

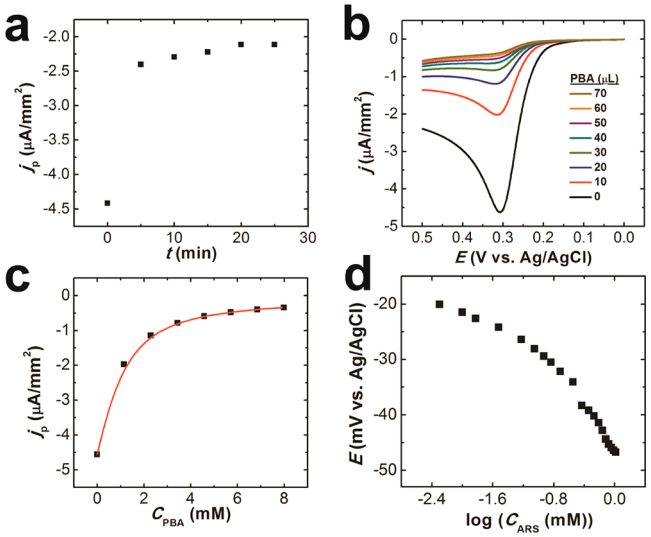


Figure 3. (a) Peak current density (P3, Figure 2a) as a function of time measured in a solution containing 0.10 M *N*-ethylmorpholine buffer (pH 8.5), 1.0 mM ARS, 1.0 mM PBA, and 0.10 M KCl. Scan rate 0.100 V/s. (b) Linear sweep voltammograms obtained in a solution containing 0.10 M *N*-ethylmorpholine buffer (pH 8.5), 1.0 mM ARS, and 0.10 M KCl and titrated with 10 μL aliquots of 1.0 M PBA dissolved in ethanol. Scan rate 0.100 V/s. The total volume of PBA added is shown in the legend. (c) Peak current density (Figure 2b) as a function of PBA concentration. The red curve is a nonlinear fit obtained using eq 1. (d) Potential measured in a solution containing 0.10 M *N*-ethylmorpholine buffer (pH 8.5) and 0.10 M KCl as a function of the logarithm of the concentration of added ARS.

The results show that the magnitude of the anodic current density decreases quickly with time, but after ∼20 min achieves a plateau. Therefore, a reaction time of 20 min was used for the following experiments to determine the dissociation constant for the ARS−PBA complex.

To determine the dissociation constant of the ARS−PBA complex, 10 μL of 1.0 M PBA dissolved in ethanol was added to 10 mL of 0.10 M *N*-ethylmorpholine buffer containing 1.0 mM ARS and 0.10 M KCl every 20 min. The resulting linear sweep voltammograms and corresponding titration curve are shown in parts b and c, respectively, of Figure 3. With increasing concentration of PBA, the anodic peak current density of the irreversible dihydroxyl oxidation (P3 in Figure 2a) decreases. The titration curve was fitted using eq 1, and the dissociation constant of the ARS−PBA complex was determined to be $(5.63 \pm 0.05) \times 10^{-4}$ M.

Potentiometric Determination of HbA1c. The detection limit of potentiometric methods can be significantly lower than that for LSV, and therefore, this approach has been used to quantify trace amounts of analytes and weak interactions between molecules. For example, Shoji et al.[28] used potentiometry to determine the concentration of saccharides on the basis of the binding of saccharides to poly-(anilineboronic acid) (PABA)-modified glassy carbon electro-

dx.doi.org/10.1021/ac3032228 | *Anal. Chem.* 2013, 85, 1834−1839

des. Like that of ARS, the electrochemistry of PABA involves both proton and electron transfers. Accordingly, complexation between saccharides and PABA shifts the redox potential due to changes in both the pK_a and the formal potential of PABA. Specifically, formation of a negatively charged complex between the diols and boronic acid increases electron density and thus shifts the formal potential negatively. However, the negative charge also decreases acidity and thus shifts the redox potential positively. In this case, the shift of the pK_a is dominant.

In analogy to the saccharide/PABA system, we hypothesized that the binding of PBA to ARS might also change the redox potential of ARS and that this could be used to measure the competitive binding of HbA1c to PBA. Accordingly, we first measured the potential of a solution containing 0.10 M *N*-ethylmorpholine buffer (pH 8.5), 0.10 M KCl, and increasing concentrations of ARS. As shown in Figure 3d, the potential of this solution shifts negatively with increasing ARS concentration. The correlation between potential and the logarithm of ARS concentration is not linear, because only half of the redox couple is present.

Next we titrated a solution of the same composition (0.10 M *N*-ethylmorpholine and 0.10 M KCl), but this time containing 0.10 mM ARS, with aliquots of 2.0 M PBA dissolved in ethanol. As shown in Figure 4, the potential shifts negatively, from 3.85

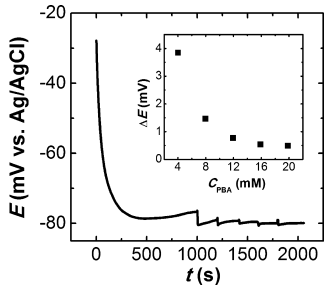


Figure 4. Potential measured in 500 μL of 0.10 M *N*-ethylmorpholine buffer (pH 8.5) containing 0.10 mM ARS and 0.10 M KCl. Starting at t = 1000 s, 1.0 μL aliquots of 2.0 M PBA in ethanol were added every 200 s. Inset: potential shifts measured immediately after each addition of PBA as a function of the PBA concentration in the solution.

to 0.49 mV, with increasing concentration of PBA. Because PBA is redox inactive, the shift in potential should be due to the formation of an ARS−PBA complex. The negative direction of the potential shift suggests that it is controlled by the change in the formal potential of the ARS complex rather its pK_a.

We hypothesized that HbA1c would compete with ARS for binding to PBA, and hence, the presence of HbA1c would alter the potential of a solution containing ARS and PBA. Accordingly, we prepared four solutions containing 0.10 M *N*-ethylmorpholine buffered at pH 8.5, 0.10 mM ARS, 0.10 M KCl, and different concentrations of HbA1c and then added 1.0 μL of 2.0 M PBA dissolved in ethanol. Figure 5 shows that the potentials of these solutions quickly shift negatively when PBA is added. This behavior is similar to that observed in the absence of HbA1c (Figure 4). However, in this case the potential slowly shifts back positively. The higher the concentration of HbA1c, the more the potential shifts, indicating that the potential shift is correlated with the competitive binding of HbA1c to PBA. Because the diffusion of HbA1c is much slower than that of ARS, due to the much larger size of HbA1c, the rate of formation of the HbA1c−PBA complex is much slower than that of the ARS−PBA complex.

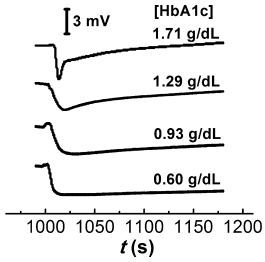


Figure 5. Potential as a function of time measured in each of four 500 μL aliquots of 0.10 M *N*-ethylmorpholine buffer (pH 8.5) containing 0.10 mM ARS, 0.10 M KCl, and 50 μL of HbA1c having the concentrations given in the legend. A 1.0 μL aliquot of 2.0 M PBA in ethanol was added to the solution at 1000 s.

To quantitatively detect HbA1c, the potential shifts at different times (shown in Figure 6a) were plotted vs the

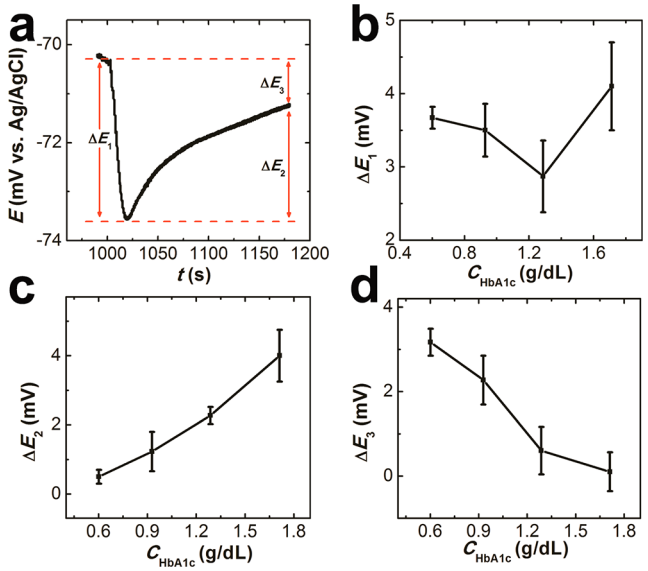


Figure 6. (a) Potential measured in 500 μL of 0.10 M *N*-ethylmorpholine buffer solution (pH 8.5) containing 0.10 mM ARS, 0.10 M KCl, and 50 μL of 1.29 g/dL HbA1c. A 1.0 μL aliquot of 2.0 M PBA in ethanol was added at 1000 s. The potential shifts at different times are labeled as ΔE_1, ΔE_2, and ΔE_3. The values of ΔE_2 and ΔE_3 are measured at 1180 s. (b) ΔE_1, (c) ΔE_2, and (d) ΔE_3 as functions of HbA1c concentration. Each ΔE value is an average of three measurements, and the error bars represent standard deviations from the mean.

concentration of HbA1c. As shown in Figures 6b,c,d, ΔE_1, which corresponds to the initial potential shift resulting from the binding of ARS to PBA, is not correlated with the concentration of HbA1c. However, ΔE_2 and ΔE_3 are clearly correlated with the HbA1c concentration. Specifically, increasing concentrations of HbA1c result in an increase in ΔE_2 from 0.5 to 4.0 mV, while ΔE_3 simultaneously decreases from 3.2 to 0.1 mV.

Determination of Total Hb. The absolute concentration of HbA1c does not necessarily represent the real value of % HbA1c, because there is a large variation in the total Hb concentration across the population.[29] Therefore, for the determination of %HbA1c, measuring the concentration of total Hb is crucial. In this study we used $Fe(CN)_6^{3-}$ as an oxidant to convert Hb to methemoglobin[30] and $Fe(CN)_6^{3-}$ to

dx.doi.org/10.1021/ac3032228 | *Anal. Chem.* 2013, 85, 1834−1839

$Fe(CN)_6^{4-}$. The resulting changes in the relative concentrations of the $Fe(CN)_6^{3-/4-}$ redox couple lead to a potential shift that is used for quantitative determination of the total Hb concentration. As shown in Figure 7a, after each addition of Hb

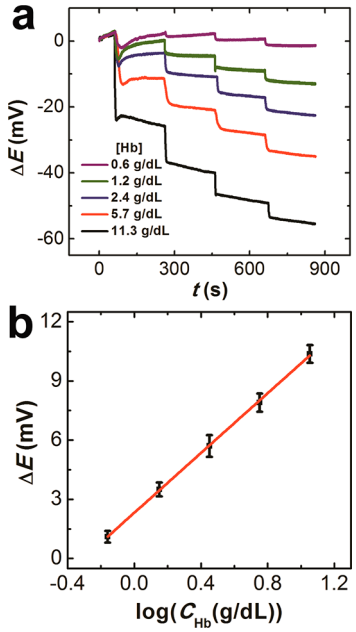


Figure 7. (a) Potential measured in 3.0 mL of 0.010 M PBS solution containing 10 mM $Fe(CN)_6^{3-}$. Aliquots (3.0 μL) of Hb, present at the concentrations shown in the legend, were added every 200 s starting at t = 60 s. The potentials in the figure are normalized to their initial values. (b) Potential shifts at t = 260 s as a function of the logarithm of the Hb concentration in the sample.

into 0.010 M PBS buffer (pH 7.4) containing 10 mM $Fe(CN)_6^{3-}$, the potential shifts quickly negatively. A linear correlation between the potential shift and the logarithm of the Hb concentration is observed (Figure 7b).

Comparison of This Potentiometric Method to Reference Values for %HbA1c. Using the potentiometric methods described thus far, the HbA1c concentration, the Hb concentration, and thus %HbA1c in four blood hemolysate samples were determined. The HbA1c concentrations were analyzed by measuring the potential shift, ΔE_2 (Figure 6a), and converting ΔE_2 to the HbA1c concentration by interpolating from the dose–response curve (Figure 6c) using the following equation:

$$C_{HbA1c} = C_{HbA1c}' + (C_{HbA1c}'' - C_{HbA1c}')\frac{\Delta E_2 - \Delta E_2'}{\Delta E_2'' - \Delta E_2'} \quad (2)$$

Here, $\Delta E_2'$ and $\Delta E_2''$ are the two ΔE_2 values, determined from Figure 6c, that bracket the measured value and C_{HbA1c}' and C_{HbA1c}'' are the corresponding HbA1c concentrations. The Hb concentrations were determined using the calibration curve shown in Figure 7b. The results obtained using the potentiometric method and a reference method (Beckman CX, provided by the sample supplier) are provided in Table 1. The %HbA1c values determined using the potentiometric method and the reference method are all within 0.5%.

Unlike immunoassays or chromatographic assays,[7,18] the method described here does not involve a washing or chromatographic separation step, which simplifies the procedure. However, it also raises the possibility that the sample matrix could bias the assay results, because interfering agents, such as diols and redox species, are abundant in blood. Importantly, however, the hemolysate standards we used for this study are sold to calibrate commercial instruments for Hb and HbA1c testing, and hence, they are representative of processed clinical samples. Furthermore, because Hb makes up 97% of the dry content of a red blood cell, most potential interferences should be present exclusively in the serum.[31] Therefore, separation of Hb from the other blood components, by centrifugation[11] or simple filtration through paper, should remove interferences that might be present in whole blood and hence interfere with this assay.[32,33]

Table 1. Assay Results Obtained Using the Reported Potentiometric Method and a Reference Method (Beckman CX)

			%HbA1c	
sample	C_{HbA1c} (g/dL)	C_{Hb} (g/dL)	potentiometric method	reference method
1	0.602	12.2	4.9	5.0
2	0.929	12.1	7.7	8.0
3	1.288	12.3	10.5	10.8
4	1.713	11.7	14.6	14.8

SUMMARY AND CONCLUSIONS

We have reported a potentiometric method for measuring the concentrations of HbA1c and Hb in human blood hemolysate and from these values %HbA1c. The key to this method is that binding of ARS to PBA through diol–boronic acid complexation leads to a negative shift of the redox potential and competitive binding of HbA1c to PBA leads to the displacement of ARS and thus a positive shift of the redox potential. This positive shift in the solution potential is linked to the HbA1c concentration. The total Hb concentration was determined by oxidizing Hb using $Fe(CN)_6^{3-}$ and measuring the potential shift arising from the reduction of $Fe(CN)_6^{3-}$. Four blood hemolysate samples were tested using this method, and the results were in good agreement with those determined by a reference method. The method reported here is simple, low cost, and thus useful for point-of-care diagnosis and management of diabetes mellitus. To further improve the analytical performance of this method, we are searching for optimal indicator molecules (i.e., a replacement for ARS) as well as integrating the sensor onto a microfluidic platform. The results of these experiments will be reported in due course.

ASSOCIATED CONTENT

Supporting Information

Additional cyclic voltammogram. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Ian Richards, Prof. Eric Anslyn, and Ms. Alexandra Gade for their help with this project. We gratefully

acknowledge financial support from the U.S. Army Research Office (Grant W911NF-07-1-0330) and the U.S. Defense Threat Reduction Agency. The Robert A. Welch Foundation provides sustained support for our research (Grant F-0032).

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