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Selective Electrochemical Sensing of Glycated Hemoglobin (HbA_{1c}) on Thiophene-3-Boronic Acid Self-Assembled Monolayer Covered Gold Electrodes

Jin-Young Park,[†] Byoung-Yong Chang,[†] Hakhyun Nam,[‡] and Su-Moon Park*,[†]

Department of Chemistry and Center for Integrated Molecular Systems, Pohang University of Science and Technology, Pohang, Gyeongbuk 790-784, Korea, and Department of Chemistry, Kwangwoon University, Seoul 139-701, Korea

We report a novel concept of sensing glycated hemoglobin, HbA_{1c}, which is now the most important index for a long-term average blood glucose level, by first selectively immobilizing it on the thiophene-3-boronic acid (T3BA) self-assembled monolayer (SAM)-covered gold electrode by a selective chemical reaction with boronic acid. HbA_{1c} thus immobilized is then detected by the label-free electrochemical impedance spectroscopic (EIS) measurements with a redox probe, an equimolar mixture of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$, present. The rate of charge transfer between the electrode and the redox probe is shown to be modulated by the amount of HbA_{1c} in the matrix hemoglobin solution due to the blocking effect caused by the binding of HbA_{1c} with boronic acid. Both the formation of a well-defined T3BA-SAM on the gold surface and the chemical binding of its boronic acid with HbA_{1c} in solution were confirmed by quartz crystal microbalance, atomic force microscopy, and EIS experiments.

Glycated hemoglobin, HbA_{1c}, generated from the Amadori rearrangement reaction[1] of glucose with the amino-terminated valine in the β-chain hemoglobin (Hb) molecule as shown in Figure 1a is the most important index presenting the long-term average blood glucose level.[2] The boronate affinity chromatography based on the esterification reaction between *cis*-diols of glucose and boronic acid (Figure 1b) has been widely used as a separation technique for HbA_{1c} from the Hb solution.[3,4] Quantification of HbA_{1c} has also been conducted by monitoring the degree of the fluorescence quenching of fluorephore-labeled boronic acid and UV absorption of Hb.[5−7] A number of other techniques such as ion-exchange chromatography,[8] immuno-turbidimetric methods,[9−12] boronated affinity chromatography,[3,4] and electrophoresis[13,14] have also been utilized for analysis or detection of HbA_{1c} after suitable treatment of blood samples. While many techniques have been used to determine HbA_{1c}, most of them are based on chromatographic separations or require separation before subjected to actual analysis. For this purpose, 3-aminophenylboronic acid bound to the solid support has been used widely in boronate-affinity chromatography.[3,15]

3-Aminophenylboronic acid has also been immobilized on gold surfaces by forming an amide bond between its amine group and a terminal carboxylic acid group of an alkylthiol molecule, which forms a self-assembled monolayer (SAM) on the gold surface.[16,17] 3-Aminophenylboronic acid thus immobilized on the gold-coated quartz crystal was then utilized for piezoelectric detection of HbA_{1c} by monitoring the decrease in the fundamental frequency of the crystal.

In the past few years, we have been developing sensors for a variety of compounds based on various SAMs on gold surfaces. These include the following: a molecular size selective sensor using cyclodextrin SAMs,[18] a glucose sensor employing cyclodextrin SAMs,[19] a polyamine sensor using a calix[4]crown-5 SAM,[20] a more reliable and reproducible DNA sensor based on a

* To whom correspondence should be addressed. E-mail: smpark@postech.edu. Phone: +82-54-279-2102. Fax: +82-54-279-3399.

[†] Pohang University of Science and Technology.

[‡] Kwangwoon University.

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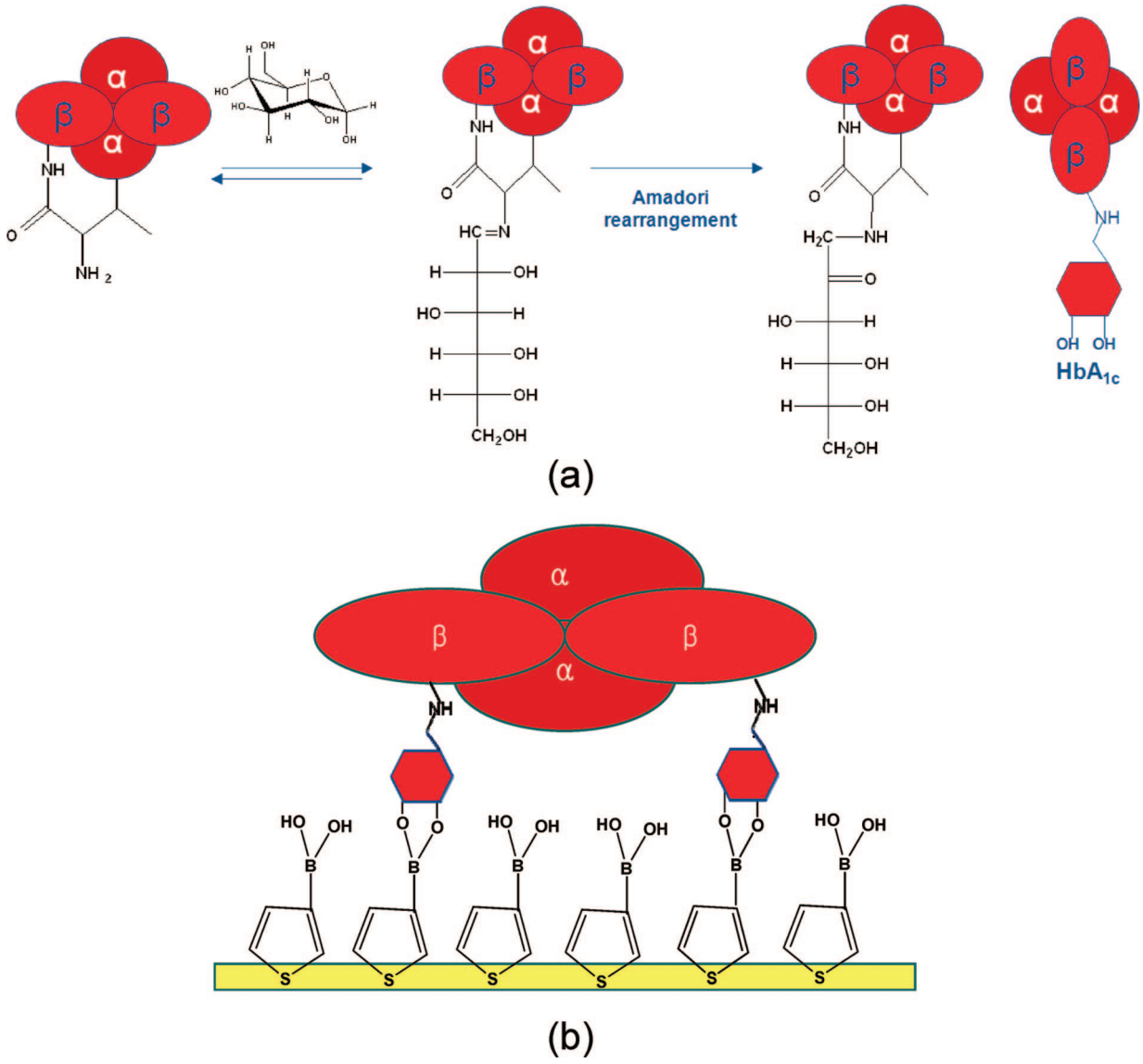

Figure 1. (a) Amadori rearrangement of glucose with amino-terminated valine in β-chain hemoglobin (taken from ref 1) and (b) the esterification reaction between glycated HbA_{1c} and boronic acid of the T3BA-SAM.

dendrone SAM,[21] and protein sensors for the C-reactive protein and ferritin employing (*R*)-lipo-diaza-18-crown-6 SAM.[22] In our present study, we use a thiophene-3-boronic acid (T3BA) SAM on a gold surface for selective analysis of HbA_{1c}. It is well-known that thiophene forms a $\pi-\pi$ stacked, well-defined SAM on the gold surface.[23,24]

EXPERIMENTAL SECTION

Materials. Standard HbA_{1c} solutions, JCCLS CRM004a, containing five different levels, 4.54 (±0.05), 5.27 (±0.06), 6.96 (±0.08), 9.24 (±0.10), and 11.58% (±0.13) of HbA_{1c}, in an Hb matrix containing 140 (±10) g/L Hb, were obtained from the Health Care Technology Foundation (Kawasaki, Japan) for calibration purposes and stored at 4 °C. These solutions were diluted with a buffer (pH 8.5) solution prepared by dissolving 10 mM 4-ethylmorpholine in water. Another HbA_{1c} standard solution, which contained 76 mg/dL HbA_{1c} in a 902 mg/dL Hb solution, was purchased from Daiichi Pure Chemicals (Tokyo, Japan). The latter, which contains 8.4% HbA_{1c} with respect to the total Hb content, was used for the detection of various concentrations of HbA_{1c} after it was diluted to appropriate concentrations. In this case, the matrix effects exerted by Hb molecules would be different as the absolute amount of Hb is varied depending on the dilution factor although the HbA_{1c} concentration with respect to Hb would stay constant regardless of the dilution factor. On the other hand, the former (JCCLS CRM004a) would display the same levels of matrix effects thanks to the same amounts of Hb. T3BA was obtained from Frontier Scientific, Inc. All the other chemicals were obtained from Aldrich. A Bioanalytical System (West Lafayette, IN) BASi MF-2014 gold disk electrode (0.020 cm^2) was used as a working electrode with a homemade Ag/AgCl electrode (in saturated KCl), and a platinum spiral wire used as a reference and counter electrodes.

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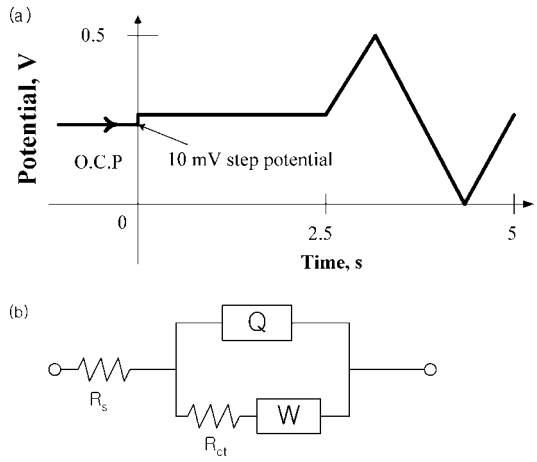

Figure 2. (a) Potential program for sequential acquisition of FT-EIS and CV data and (b) an equivalent circuit for analysis of impedance data. R_s, R_{ct}, Q, and W represent the solution resistance, charge-transfer resistance, constant-phase element (CPE), and Warburg impedance, respectively. The dispersion of the capacitor at the SAM-covered surface is described by the CPE, Q.

Quartz Crystal Microbalance (QCM) and Atomic Force Microscopic (AFM) Experiments. A Biomechatron model 1000 L EQCN (Jeonju, Korea) electrochemical quartz crystal analyzer (EQCA) was utilized for measuring the amount of T3BA chemisorbed on a gold-coated, AT-cut QCM electrode, which had a fundamental frequency of 9.0 MHz and an area of 0.196 cm^2. The decrease in frequency was monitored upon injection of a 100-μL aliquot of 10 mM T3BA in methyl alcohol (MeOH) into 2.0 mL of MeOH in the QCM cell. The sensitivity of the EQCA was 1.41 ± 0.02 ng/Hz for the above electrode by calibration with the silver deposition reaction. After T3BA was immobilized on a gold-coated QCM electrode, it was washed thoroughly with MeOH and dried by purging with N_2 gas. In the stabilized QCM cell having 2.0 mL of the buffer solution (pH 8.5) made of 10 mM 4-ethylmorpholine, a 100-μL aliquot of the HbA_{1c} solution, which had been diluted to 1/100 of the original solution containing 11.6% HbA_{1c} with respect to the amount of Hb, was injected, and the decrease in frequency was monitored.

In addition to the QCM experiments, an AFM image was obtained to visually confirm the immobilized HbA_{1c} molecules on

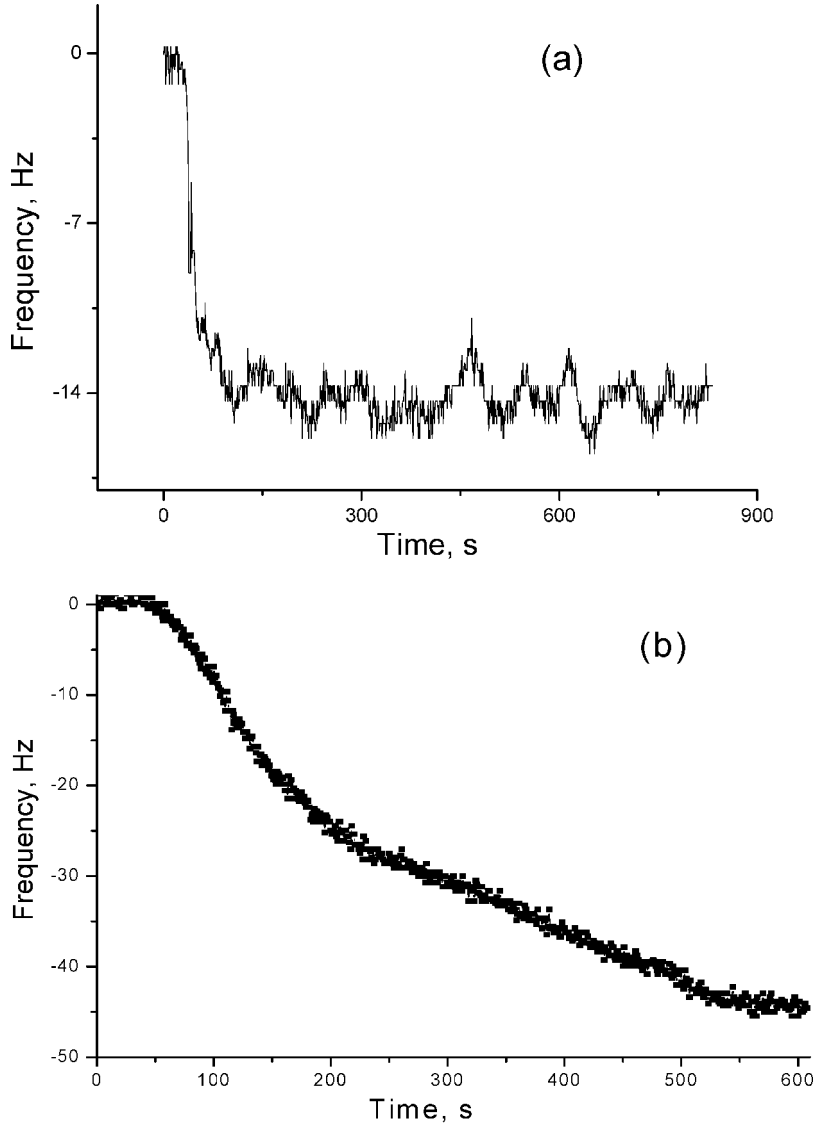

Figure 3. QCM results for (a) the immobilization of the T3BA-SAM and (b) the HbA_{1c} binding upon injection of 100 μL of diluted 11.6% HbA_{1c} solution into 2 mL of the pH 8.5 buffer solution (10 mM 4-ethylmorpholine).

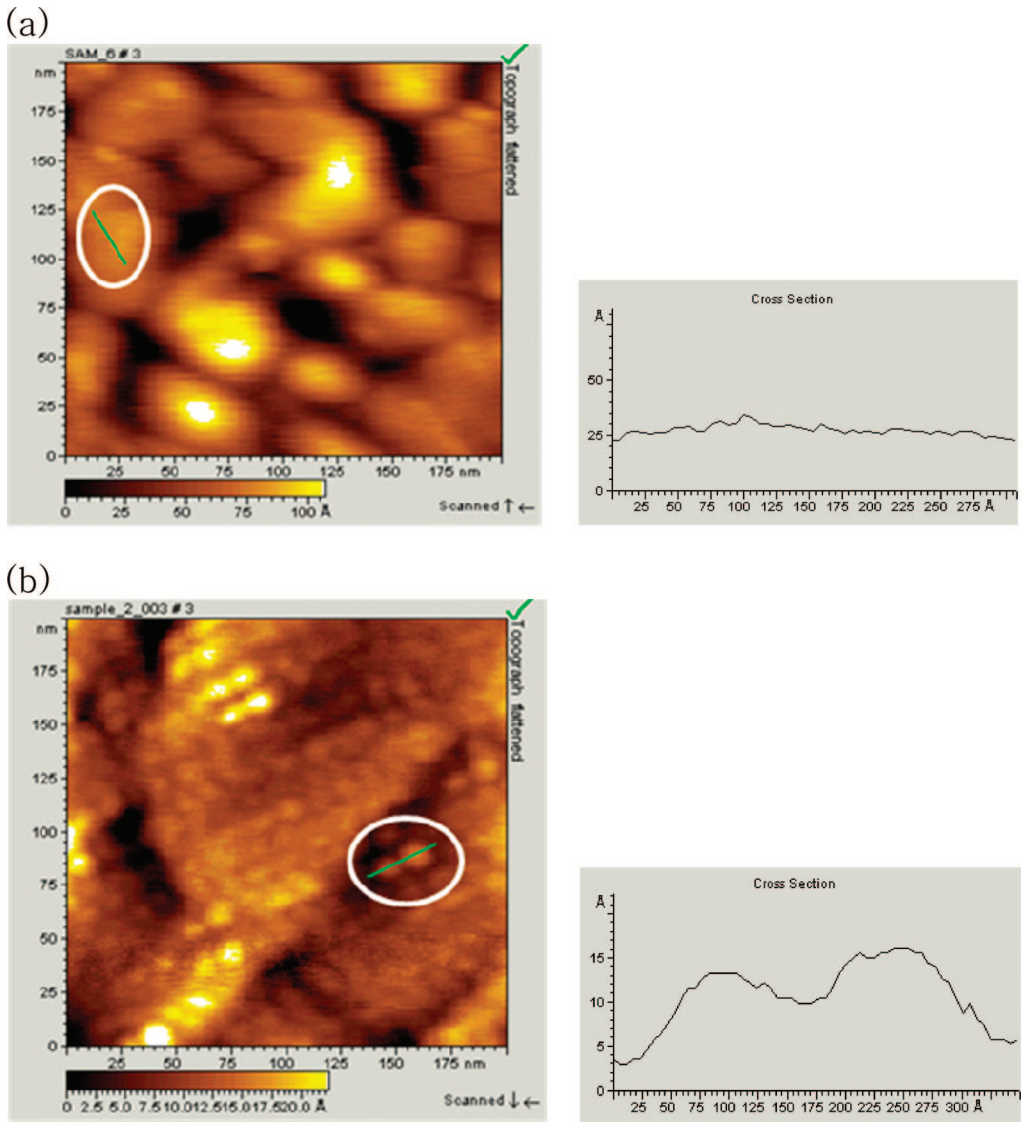

Figure 4. AFM images of (a) the thermally annealed bare gold-on-silicon surface and (b) the HbA_{1c}/T3BA-SAM immobilized on it along with corresponding cross-sectional profiles of the spots marked by white circles on the images.

the T3BA-SAM. A gold-on-silicon electrode with a thickness of gold of 200 Å (Inostek, Ansan, Korea) was used as an AFM substrate after it was cleaned in a piranha solution (70% H_2SO_4/30% H_2O_2) for 1 min and annealed with a H_2 flame for 3 min to expose single crystal-like facets. The annealed substrate was then immersed in a 10 mM T3BA solution in MeOH for 3 h. Then, a 200-μL aliquot of 76 μg/mL HbA_{1c} (in 902 μg/mL Hb) solution was dropped on the substrate covered by the T3BA-SAM for 10 min to allow HbA_{1c} to be immobilized on the T3BA-SAM. The PicoSPM MAC mode AFM (Molecular Imaging Inc., Tempe, AZ) was used for imaging the topography of HbA_{1c} molecules thus immobilized. The cantilever had a force constant of 2.8 N/m with a resonance frequency of 75 kHz, and the tip was scanned at a speed of 2.5 ms/nm.

Electrochemical Impedance Spectroscopy (EIS) Measurements. A gold disk electrode was cleaned by polishing sequentially with alumina powder of 14.5, 5, 1, 0.3, and 0.05 μm, and then the cleanness of the surface was confirmed by cyclic voltammetry in 1 M H_2SO_4. The cleaned gold disk electrode was dipped in a 10 mM solution of T3BA dissolved in MeOH for 3 h. The impedance measurements were made in a solution containing 2.5 mM each $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$, 0.25 M KCl, and 0.10 M NaCl dissolved in a pH 8.5 buffer solution prepared by dissolving 10 mM 4-ethylmorpholine in water. The T3BA-SAM-covered electrode was then exposed to solutions containing various levels of HbA_{1c} for EIS measurements.

Impedance data were acquired using a Solartron model SI 1255 HF frequency response analyzer connected to an EG&G 273 potentiostat/galvanostat at an open circuit potential, 0.245 V versus Ag/AgCl (in saturated KCl), taken as a dc bias potential, on which an ac wave of 5 mV (peak-to-peak) was overlaid in a frequency range of 100 kHz−100 mHz. The data acquisition was made at a rate of five points per decade, and the measurements were stopped when the impedances began to be affected by mass transfer, as the mass transfer at the low frequency offered no significance in our measurements. The data thus obtained were fitted to an appropriate equivalent circuit using the ZsimpWin (Princeton Applied Research) program.

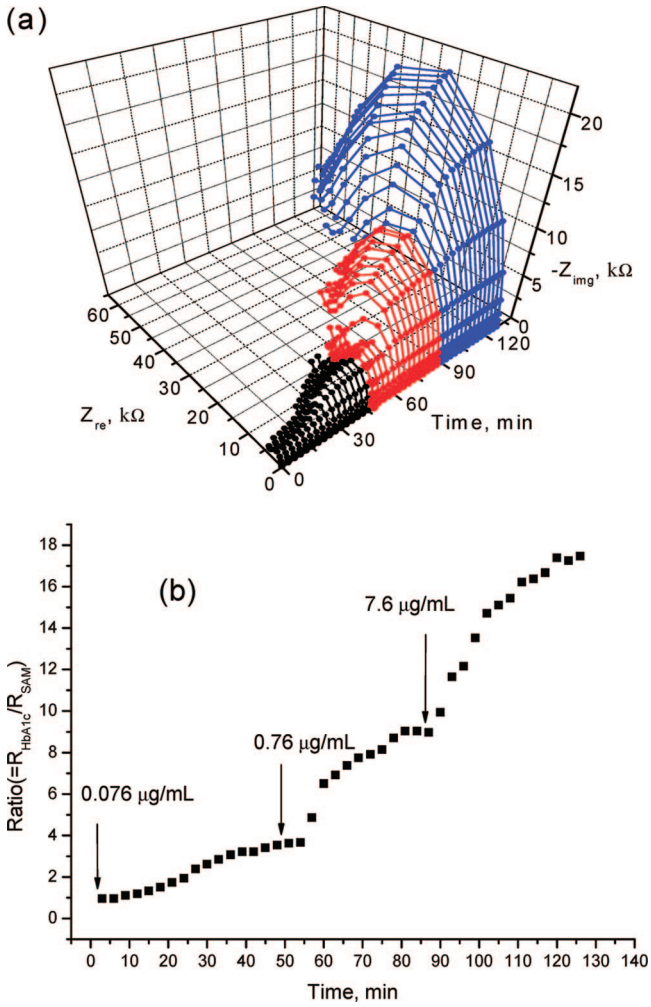

Figure 5. (a) Impedance data obtained upon sequential injection of 40 μL of 7.6, 76, and 760 μg/mL HbA_{1c} solutions (in 9.02 mg/mL Hb) to 4.0 mL of the pH 8.5 buffer solution containing 2.5 mM each $Fe(CN)_6^{3-/4-}$, 0.50 M KCl, 0.10 M NaCl, and 10 mM 4-ethylmorpholine. The data were obtained by the FRA method at a dc bias potential of 0.245 V with an ac voltage of ±5 mV overlaid for the frequency range of 100 kHz−1 Hz. (b) R_{ct} ratios (= R_{HbA1c}/R_{SAM}) for the data shown in (a).

Impedance measurements were also made employing our recently developed Fourier transform (FT) EIS technique.[25−30] A homemade fast-rise potentiostat, which has a rise time of <50 ns/V, was utilized for the FT-EIS measurements. The programmed potential function shown in Figure 2a was generated by an Agilent 33120 Arbitrary Waveform Generator and was inputted into the working electrode via the potentiostat every 5 s. Impedance data were obtained during the first 2.5 s using a potential step of 10.0 mV of 2.5 s, which corresponded to the lowest frequency of 0.40 Hz. The FT-EIS method of data acquisition, and the data treatment have been described in detail elsewhere.[27−30] The impedance data were then calculated in a frequency range of 10 kHz−0.40 Hz from the chronoamperometric data obtained upon stepping the potential of 10.0 mV. During the next 2.5 s, a cyclic voltammogram (CV) was recorded employing the potential sweep at a scan rate of 400 mV/s. For the data acquisition, a National Instrument NI-5922 high-speed data acquisition card with 24-bit resolution plugged into a PCI slot of a Pentium-4 PC was used. All the data were recorded at a rate of 100 KHz upon application of the step or sweep signals. With the FT-EIS experiments, changes in charge-transfer resistances was monitored upon injecting 40 μL of each level of the HbA_{1c} stock solution (JCCLS CRM004a) into 4 mL of the pH 8.5 buffer solution, which contained the aforementioned redox probe and supporting electrolytes. Impedances were then computed by the Matlab program by first taking the first derivative of the stepped voltage and the chronoamperometric current obtained thereof, followed by transforming the data from the time domain into the frequency domain in the frequency range of 0.4 Hz−10 KHz. The procedure for the data acquisition and the algorithm for impedance calculation has been described elsewhere in detail.[25−30] Nyquist plots thus obtained were fitted to the equivalent circuit shown in Figure 2b using the ZsimpWin (Princeton Applied Research) program.

RESULTS AND DISCUSSION

Immobilization of HbA_{1c} on the Thiophene-3-Boronic Acid SAM. We first conducted QCM and AFM experiments to confirm whether T3BA forms a well-defined SAM on the gold surface, which would selectively recognize the HbA_{1c} protein. The amounts of the T3BA-SAM formed on the gold surface and of HbA_{1c} immobilized on the SAM were quantitatively monitored by QCM experiments. As shown in Figure 3a, the decrease in frequency was −14 Hz upon injection of 100 μL of the T3BA solution in MeOH to 2.0 mL of MeOH in the QCM cell. The frequency decrease of −14 Hz corresponds to 4.8×10^{14} molecules/cm^2. It has been reported that the sulfur atom of the π-stacked thiophene SAM is bonded to the gold atom with a space of 5 Å on Au(111),[23] which translates into a molecular density of 4.0×10^{14} molecules/cm^2, in excellent agreement with ours. Thiophene has been shown to form a well-defined SAM on gold surfaces by a few investigators[23,24,31] in direct conflict with theoretical predictions by Elfeninat et al.[32] Ours is ~20% greater than that calculated from Dishner et al.'s observation by scanning tunneling microscopic imaging experiments,[23] which is reasonable considering the roughness of the polycrystalline gold surface of the QCM electrodes, which have been prepared by vapor deposition methods. Thus, T3BA must have formed a dense and well-defined SAM on the gold surface with a compact structure.

We also conducted QCM experiments in order to confirm whether HbA_{1c} is selectively recognized by the T3BA-SAM. After 100 μL of the HbA_{1c} solution, which was prepared by diluting the original reference solution containing 11.6% HbA_{1c} (one of JCCLS CRM004a in the 140 g/L Hb matrix) to 1/100 with the buffer solution, was injected to 2.0 mL of the stabilized buffer solution in the QCM cell containing the SAM-covered QCM electrode, the frequency decrease was determined to be −44 Hz in ~10 min (Figure 3b). The reaction between the boronic acid on the electrode surface and the HbA_{1c} protein is not very fast as can be

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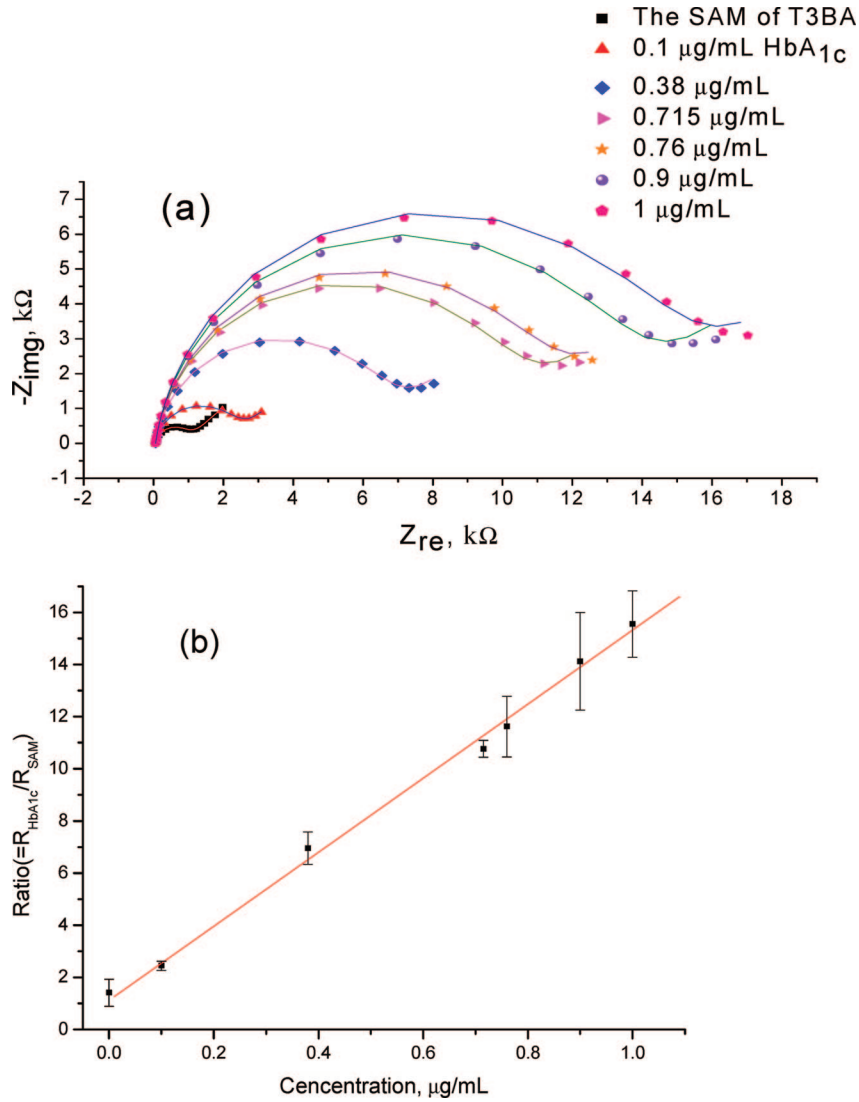

Figure 6. (a) Impedance data obtained for the T3BA-SAM-covered electrode before and after it was dipped into the various concentrations of HbA_{1c} diluted with 10 mM 4-ethylmorpholine buffer (pH 8.5) for 5 min. (b) The ratio of resistances plotted versus log(concentration of HbA_{1c}, μg/mL).

seen from Figure 3b, taking about three steps to its completion in ~10 min. The frequency eventually decreases by −44 Hz, which corresponds to the molecular density of 3.0×10^{12} molecules/cm^2. The stability of the immobilized HbA_{1c} was confirmed by both QCM and EIS experiments; the minimum frequency remained the same when the HbA_{1c} solution was replaced with a pure buffer solution. This was also confirmed by impedance measurements as well, as will be described below.

The image of the surface of the annealed gold-on-silicon electrode, on which HbA_{1c} molecules had been immobilized by their reaction with boronic acids on the T3BA-SAM, was recorded with an AFM. To prepare the surface for the image recording, we first allowed the HbA_{1c} molecules to immobilize for 5 min on the top of the T3BA monolayer by dropping 200 μL of the 76 μg/mL HbA_{1c} solution (in 902 μg/mL Hb) onto the SAM-covered gold-on-silicon surface. The electrode was then washed thoroughly with water, dried, and then subjected to the AFM examination. Figure 4 shows AFM images of the following: (a) the T3BA-SAM on annealed bare gold surface and (b) the one with HbA_{1c} molecules immobilized along with their height profiles on their right. When the gold-on-silicon is thermally annealed by a hydrogen flame, surfaces of gold grains become atomically flat, allowing scanning probe microscopic examination to be made. We clearly see gold grains of their diameters ranging 35−55 nm in Figure 4a, one of whose surface is shown to be atomically flat as displayed by its height profile. Figure 4b clearly shows two isolated HbA_{1c} molecules, whose widths are ~15 nm with their heights of about 13−15 Å, which is consistent with the protein size reported in the literature.[33] Thus, the T3BA-SAM captures the HbA_{1c} molecules to form rather nicely organized arrays of spheres. As was shown in our earlier work,[22] bare and SAM-covered gold surfaces are generally indistinguishable in AFM images. Based on the QCM and AFM results, we conclude that the gold surface modified with the T3BA−SAM serves as a satisfactory platform for the immobilization of HbA_{1c} protein molecules.

Detection by Impedance Measurements. Many analytical techniques such as electrophoresis,[13,14,16] high-pressure ion-

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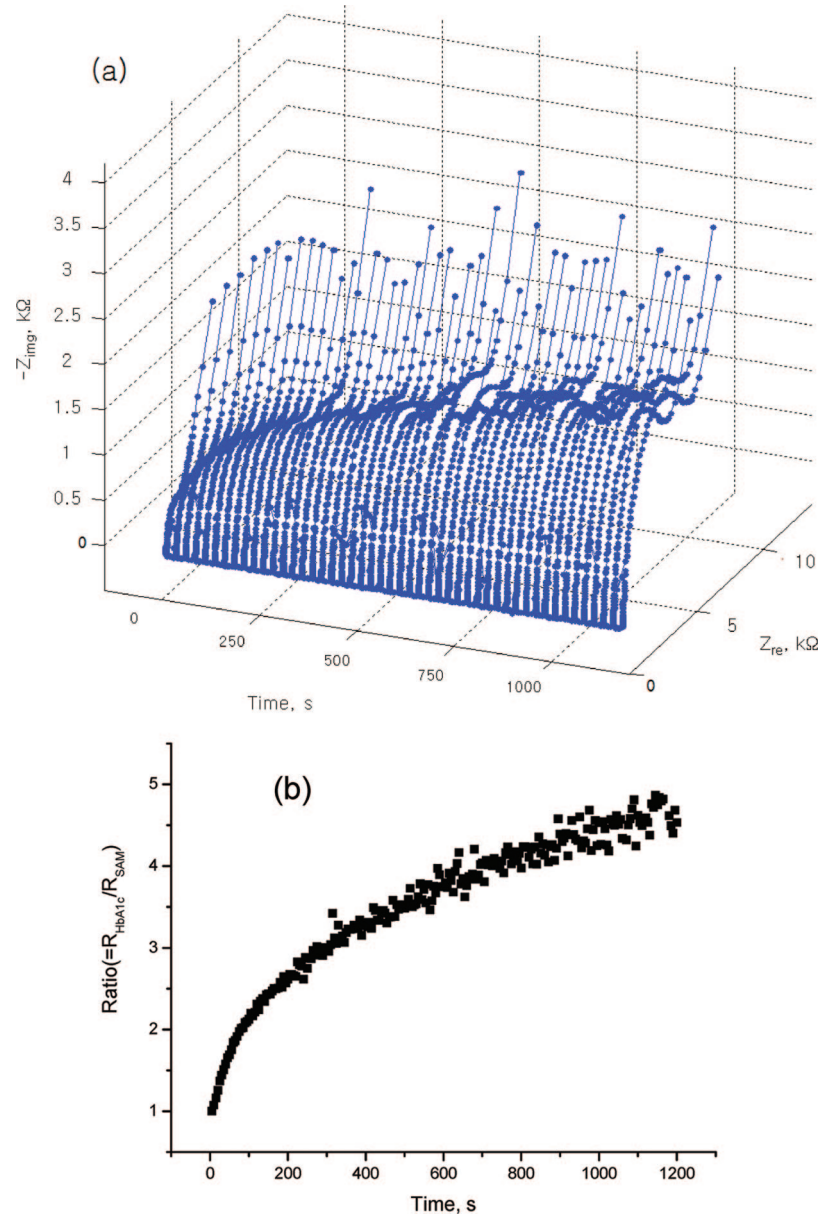

Figure 7. (a) Series of Nyquist plots obtained upon injection of 40 μL of the 6.96% HbA_{1c} solution to 4.0 mL of the same buffer solution as used for Figure 5. The data shown were obtained every 25 s although the raw data were obtained every 5 s. (b) The R_{ct} ratio plotted for the data shown in (a).

exchange liquid chromatography,[8,34] immunoassay,[10−12,35,36] and fluorescence quenching[7,15,37] have been developed for the determination of HbA_{1c}. An electrochemical technique has been used to determine various ranges of the HbA_{1c} fraction over total Hb,[17,38,39] in which the HbA_{1c} molecules were allowed to react with the human antibody (immunogloblin-G, IgG) labeled with an electroactive group, ferrocenecarboxylic acid (Fc-IgG), and the reaction product was detected in a flow cell by an amperometric method at an applied potential of 600 mV versus Ag/AgCl electrode. This procedure required a reaction of HbA_{1c} with the prelabeled Fc-IgG for the amperometric detection after the chromatographic separation.

In our present study, we used a label-free EIS detection method as the selective immobilization of the analyte, HbA_{1c}, had already been achieved by the reaction with boronic acid on the T3BA-SAM-covered surface. The selective immobilization of HbA_{1c} on the gold surface would modulate the capability of the gold electrode for electron transfer by blocking the electrode surface and, thus, allows the amount of the analyte to be determined. The changes in impedances were first monitored as a function of time to see if the HbA_{1c} molecules interact with boronic acid on

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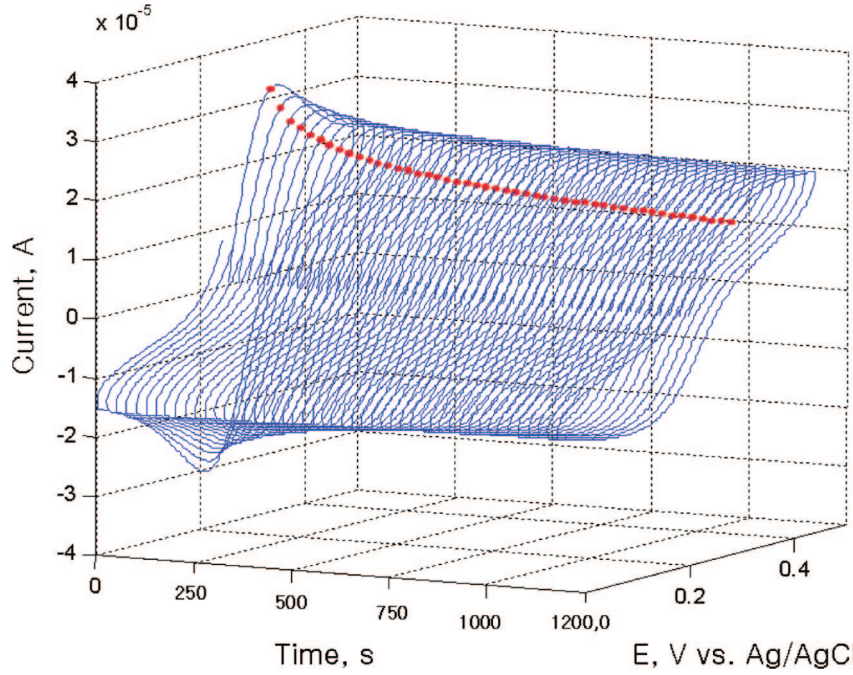

Figure 8. Series of cyclic voltammograms recorded every 25 s along with acquisition of impedance data shown in Figure 7. The peak currents are marked by red filled circles.

the T3BA-SAM by the conventional frequency response analysis method. A full impedance spectrum was recorded in a frequency range of 100 kHz−1 Hz every 3 min using the frequency response analysis (FRA) method, and the results obtained upon sequential addition of 40 μL of the HbA_{1c} solution containing 7.6 (in 90.2 μg/mL Hb), 76 (in 902 μg/mL Hb), and 760 μg/mL (in 9.02 mg/mL Hb) are shown in Figure 5a. The impedance data are satisfactorily described by the equivalent circuit shown in Figure 2b, which contains just one *RQ* time constant with *Q* being a constant-phase element. When the surface has the T3BA-SAM only, the charge-transfer resistance, R_{ct}, for electron transfer to and from the redox probe ions, 2.5 mM $Fe(CN)_6^{3-/4-}$, in the electrolyte solution containing 0.50 M KCl and 0.10 M NaCl at pH 8.5 was in the range of a few kiloohms, indicating that the SAM has relatively good electrochemical activity due perhaps to the π-conjugated thiophene system covering the electrode surface. Further, the fact that the impedance data are described by a single semicircle indicates that the surface of the T3BA-SAM is uniform and well-defined with no other defects or routes available for electron transfer. The *Q* element has *n*-values close to ~0.9 (not shown), suggesting that the SAM has characteristics for a typical capacitor with some inhomogeneities present on the surface. Upon injection of 40 μL of 7.6 μg/mL HbA_{1c} into 4.0 mL of the electrolyte in the electrochemical cell, which makes a final HbA_{1c} concentration of 0.076 μg/mL, the R_{ct} value was increased to about three times that of the initial value by the time the reaction was completed (Figure 5) in ~12 min. For the analysis of HbA_{1c}, ratios of the charge-transfer resistances obtained for the SAM-covered electrode with and without HbA_{1c}, i.e., $R_{HbA1c\text{-}SAM}/R_{SAM}$, were used (Figure 5b).[20−22] The data shown in Figure 5a are normalized charge-transfer resistances with respect to that at the SAM-covered electrode without HbA_{1c}, i.e., $R_{HbA1c\text{-}SAM}/R_{SAM}$, in Figure 5b. When the HbA_{1c} concentration is further increased, the R_{ct} values increased correspondingly as shown in Figure 5b.

We also evaluated the stability of immobilized HbA_{1c} by impedance measurements for prolonged periods. Reproducible results were obtained from the immobilized HbA_{1c} layer when the solution was allowed to stand for a few hours with or without replacing the buffer solution with another (data not shown), which is consistent with the QCM results. Also, reproducible results were obtained even if SAMs were modified from different batches at electrodes of different areas as long as the ratios of the charge-transfer resistances, i.e., $R_{HbA1c\text{-}SAM}/R_{SAM}$'s, were used instead of absolute resistance values to relate with analyte concentrations. We have examined these effects in detail in our earlier work;[21] this approach solved a long-standing problem of irreproducibility encountered in analysis using SAMs.

Figure 6 shows how the R_{ct} values change upon increasing the HbA_{1c} concentration, and the ratio of the R_{ct} values in the presence of varied HbA_{1c} concentrations to that with only T3BA-SAM present is linear. Normally, the ratio is linear for a small dynamic range of the analyte concentration but can become exponential as the range becomes wider due to the nonlinearity of the electrochemical current with the overpotential.[22] Note also that the results shown in Figures 5 and 6 were obtained from the solutions, in which concentrations of HbA_{1c} and hemoglobin were diluted by the same factor and the relative concentration of HbA_{1c} with respect to Hb remained the same regardless of the dilution factor. It should be pointed out that Figure 6b also includes the data points obtained from a T3BA-SAM-modified electrode in solutions containing a larger amount of Hb (20 μg/mL) than in any sample solutions without any HbA_{1c} present.

In order to compare the changes in impedance data with those in cyclic voltammetric peak currents upon injection of HbA_{1c} recorded in real time, we also ran FT-EIS/CV combined experiments using the programmed potential shown in Figure 2a. This experiment allows both the impedance and CV data to be obtained sequentially, and the FT-EIS measurement was made first via the

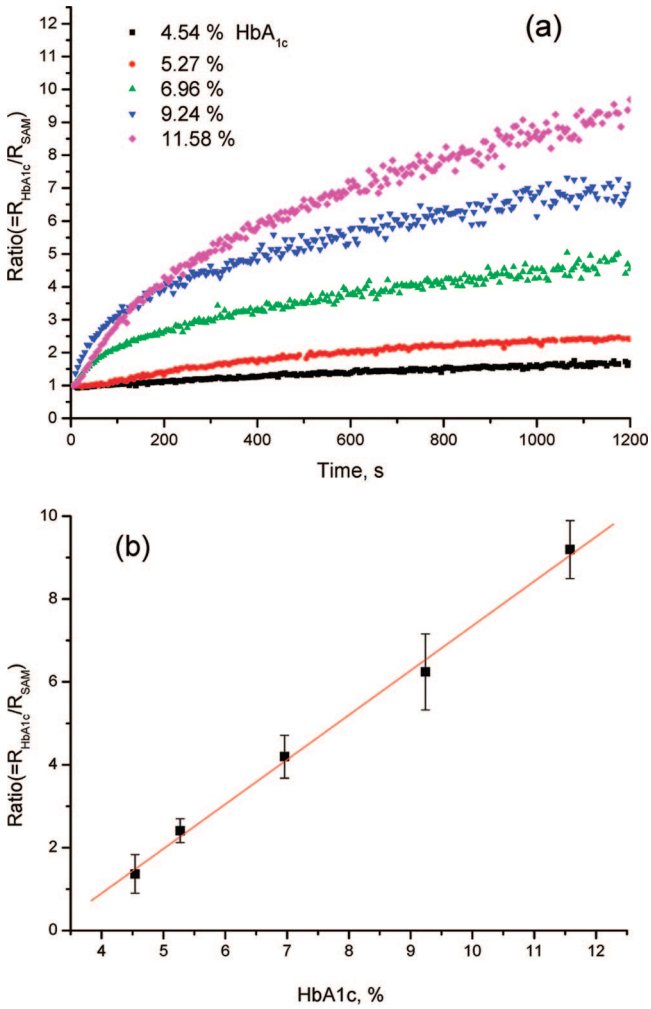

Figure 9. (a) Impedance data obtained for 1200 s upon injection of 40 μL of reference HbA_{1c} solutions containing 4.54, 5.27, 6.96, 9.24, and 11.58% HbA_{1c} with respect to Hb (140 g/L). (b) The R_{ct} ratio plotted for five different HbA1c levels.

10-mV step at the open circuit potential established by the equimolar mixture of 2.5 mM $Fe(CN)_6^{3-/4-}$ (0.245 V versus Ag/AgCl in saturated KCl) for the first 2.5-s period and then a full CV was recorded for the next 2.5 s at a scan rate of 400 mV/s. The series of impedance data obtained for a reaction period of 20 min upon addition of 40 μL of 6.96% HbA_{1c} in 140 g/L Hb (one of JCCLS CRM004a) are shown in Figure 7 and corresponding CVs in Figure 8. As can be seen from the CVs shown in Figure 8, the peak current decreased to 46.5% of its initial value with HbA_{1c} present over 20-min period due to the progression of the reaction. However, the resistance increased by ~480% in the meantime, which clearly displays that the impedance measurements provide much more sensitive signal responses than the current measurements as we described in our earlier study.[22] This is because the current is related exponentially with the potential for an electrochemical reaction via the Butler−Volmer equation.

Finally, the primary reference solutions (JCCLS CRM004a by the Japanese Committee for Clinical Laboratory Standards) containing five different levels of HbA_{1c} ranging 4−13% for a given amount of hemoglobin (in 140 ± 10 g/L) were utilized to see how the HbA_{1c} would respond to the T3BA-SAM-covered electrode when a large amount of Hb is present as a matrix. While the results shown in Figures 5 and 6 were obtained from the reference solutions having an identical HbA_{1c} content (8.4%) with respect to Hb no matter how much the reference solution was diluted, the JCCLS CRM004a reference solutions have different ratios of HbA_{1c} to Hb. These references were prepared in the same way as for chromatographic analysis of HbA_{1c} by first centrifuging human whole blood and removing erythrocyte ghost membranes, followed by adding a carbonate buffer solution and dialysis.[40] The impedance data obtained over a 20-min period using these reference solutions are shown in Figure 9a upon injection of 40 μL of these reference solutions to 4.0 mL of solution containing the redox probe ions and other necessary electrolytes at pH 8.5. The signal keeps increasing even after 20 min without being saturated as was the case for Figure 7. This is because the reaction is not completed within this period. Nevertheless, the ratios of the R_{ct} values obtained in the presence of HbA_{1c} to that without it, which are obtained at 20 min after the sample injection, increase linearly with the increase in the HbA_{1c} concentration as can be seen in Figure 9b. When the level of HbA_{1c} increases up to 11.58%, the R_{ct} value increases to the 9.69 times of the initial R_{ct} value. The linearity of the increasing ratio of R_{ct} values versus the HbA_{1c} level indicates that the T3BA-SAM-modified electrode provides a satisfactory platform for sensing HbA_{1c}. Further, we have also confirmed that the R_{ct} values obtained at earlier periods gave good linearity although the sensitivity represented by the slope of the plot is lower (results not shown). Results of these experiments indicate that the responses of the T3BA-SAM to HbA_{1c} are linear irrespective of relative amounts of Hb present and the time at which the impedance signals are taken although the sensitivity is different. Finally, it should be pointed out that the SAM-modified electrodes can be used repeatedly after washing with a sodium acetate buffer of pH 5, which reverses the immobilization reaction for HbA_{1c} as is the case for the chromatographic column. We have confirmed the reusability by this procedure. We also point out that the FT-EIS measurement offers a possibility of sensing and analyzing the level of HbA_{1c} proteins in a much shorter time than any other known techniques.

The method we describe here may not be regarded to be specific for glycated HbA_{1c} in blood as other glycated proteins such as gHSA, HbA_{1c}, and HbA_{1b} are also present. However, glycated HbA_{1c} is a major component of these, which is why its content has been used for evaluation of the long-term sugar level in blood. Also, albumin, which is present in a large quantity in blood, is known to be glycated under hyperglycemic conditions. However, glycated albumin is separated from the red blood cells containing hemoglobin by centrifugation, and the red blood cells thus obtained are then treated by the carbonate buffer and dialyzed before being subjected to this analysis. Thus, glycated albumin is removed during the pretreatment stage of the sample, which would otherwise be determined by the same method as used here.[41]

CONCLUSIONS

We have demonstrated the formation of the well-defined T3BA-SAM by QCM, AFM, and EIS experiments. Boronic acid on the

(40) Reference Material Institute for Clinical Chemistry Standards (ReCCS) manual, Kanagawa Science Park A205, 3-2-2 Sakado, Takatsu-ku, Kawasaki-shi, 213-0012, Japan.

(41) Ikeda, K.; Sakamoto, Y.; Kawasaki, Y.; Miyake, T.; Tanaka, K.; Urata, T.; Katayama, Y.; Ueda, S.; Horiuchi, S. *Clin. Chem.* **1998**, *44*, 256.

SAM thus formed was shown to selectively react with the HbA_{1c}, and the level of HbA_{1c} can thus be selectively determined using EIS measurements. To our knowledge, this is the first demonstration of the formation of the T3BA-SAM and its application to the HbA_{1c} sensor under a variety of different experimental conditions. The selective reaction of HbA_{1c} with T3BA on its well-defined SAM-covered electrode in an appropriate buffer solution containing redox probe ions along with other supporting electrolyte eliminates the separation step for its sensing and quantification. In addition to the conventional method of impedance measurements, we also used the FT-EIS technique for impedance measurements to see if faster methods of EIS detection can be developed with simpler equipment. The FT-EIS experiments were shown to be applicable to the studies of the reaction kinetics and to the faster real-time detection of analytes. In addition to the detection of HbA_{1c}, the FT-EIS method can be widely used for speedy detection of numerous biological interactions taking place at electrode/electrolyte interfaces.

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