

SPCE Based Glucose Sensor Employing Novel Thermostable Glucose Dehydrogenase, FADGDH: Blood Glucose Measurement with 150nL Sample in One Second

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Abstract

Background:

Self-monitoring of blood glucose (SMBG) is an important component of the modern therapy for diabetes mellitus. Thanks to the current progress in electronics and sensor fabrication technology, both the time and the blood sample volume required for the measurement have decreased drastically. However, devices that work with an even smaller sample volume and a shorter measurement time are in demand.

Methods:

A disposable glucose sensor that works with an ultra-small sample volume was developed employing the novel thermostable glucose-dehydrogenase (FADGDH) complex composed of a catalytic subunit, an electron transfer subunit (cytochrome c), and a small subunit. The electrode is a screen-printed carbon electrode (SPCE), and hexaammineruthenium (III) chloride (Ru complex) is utilized as the electron mediator. A disposable enzyme sensor was constructed by depositing the FADGDH complex and Ru complex onto the SPCE, and the sensor performance was evaluated.

Results:

Whole-blood glucose can be measured within 1 sec using this enzyme sensor and a 150-nL whole-blood sample, with high precision (>0.99) and high reproducibility ($CV < 0.45\%$) within the glucose concentration range of 0-533 mg/dL. The sensor reading was stable for more than 60 days even at 70°C.

Conclusions:

The simplicity of the construction and the high precision of this FADGDH-based glucose biosensor makes it an alternative to previously reported commercially available glucose sensors. Especially the sample volume of 150 nL and the 1-sec measurement time are the highest specifications in the world for currently available glucose sensors designed for the SMBG.

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Abbreviations: (CV) coefficient of variation, (PQQGDH) pyrroloquinoline quinone glucose dehydrogenase, (R) correlation coefficient, (SPCE) screen-printed carbon electrode, (SMBG) self-monitoring of blood glucose, (TB) time base

Keywords: catalytic, glucose, glucose-dehydrogenase (FADGDH), recombinant, sensor

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Introduction

The SMBG is an important component of the modern therapy for diabetes mellitus. SMBG has been recommended to people with diabetes as well as the health care professionals responsible for their care so that they can achieve precise glycemic control and prevent hypoglycemia.¹⁻⁶ In light of this, portable, handheld devices that measure the blood glucose level have been produced, whereby diabetics can easily and conveniently measure the blood glucose level even when they are away from home.⁷⁻¹² The currently available glucose monitoring devices are based on enzymatic reactions, combined with either electrochemical detection or color-developing reactions. Thanks to the current progress in electronics and sensor fabrication technology, both the time and the blood sample volume required for the measurement have decreased drastically.¹³ Together with the introduction of alternate-site testing, diabetics are being relieved of the pain and troublesome procedure of SMBG. However, devices that work with an even smaller sample volume and a shorter measurement time are in demand.

It is important to remember that there are still a limited number of enzymes suitable for enzymatic glucose sensing systems; two such enzymes are glucose oxidase and pyrroloquinoline quinone glucose dehydrogenase (PQQGDH). Glucose oxidase has been utilized widely as the main enzyme for glucose sensing systems; however, glucose oxidase utilizes oxygen as the electron acceptor, which limits the future advance of electrochemically based glucose sensing systems. Therefore, PQQGDH has been utilized as the ideal enzyme for the electrochemical measurement of glucose. Although its substrate specificity and stability are inferior to those of glucose oxidase, its insensitivity to oxygen and its extremely high catalytic activity make this enzyme an attractive molecule for the development of micro-volume, rapid-response glucose sensors.

The authors have been engaged in the screening and engineering of enzymes suitable for glucose sensor development. We previously reported on the screening of thermostable cofactor-binding glucose dehydrogenase from a soil bacterium, *Burkholderia cepacia*. This GDH does not have PQQ as its cofactor, but it possesses FAD in its catalytic subunit. The catalytic subunit alone, FADGDH, does show dye-mediated GDH activity, with pronounced thermal stability. But this enzyme is originally an oligomeric enzyme, a FADGDH complex composed of a catalytic subunit harboring FAD in its redox center (the

α subunit), an electron transfer subunit, a covalently attached multi-heme harboring cytochrome *c* (the β subunit), and a small subunit with unknown function (the γ subunit).¹⁴⁻¹⁷ This enzyme shows high thermostability and electron transfer ability. Previously, we have reported on the construction of a flow injection analysis system employing FADGDH, utilizing enzyme samples prepared from the original strain. Recently, we have succeeded in cloning these genes and in functionally expressing the FADGDH complex.¹⁸ Therefore, the FADGDH complex is now available as a recombinant enzyme. Its catalytic activity is similar to or even higher than that of PQQGDH, due to the presence of the electron transfer subunit, which facilitates the electron transfer between the active-site cofactor and the artificial electron mediator. Moreover, the FADGDH complex shows superior thermal stability in its native environment, as well as tolerance of several organic solvents.

In this paper, we report the construction of a glucose enzyme sensor based on a screen-printed carbon electrode (SPCE), employing the newly developed FADGDH complex as the enzyme. Thanks to the high catalytic activity and stability of this enzyme, blood glucose monitoring within 1 sec using a 150-nL sample is possible, with very high accuracy, reproducibility and precision.

Materials and Methods

Chemicals

The FADGDH complex (hereinafter FADGDH) (1490 U/mg) was purified from recombinant *Escherichia coli*, as described previously.¹⁸ Hexaammineruthenium (III) chloride (Ru complex), ACES and CHAPS were purchased from Dojin Chemical (Japan), and α -D-Glucose was purchased from Nacalai Tesque (Japan).

Biological samples

The biological samples for glucose measurement were prepared from human venous whole blood. The collected blood was decanted in a heparin-Na vacuum blood collection tube (5 mL) and the hematocrit was adjusted to 42%. The collected sample was turned upside down and agitated to prevent hemolysis, and a glucose solution was added in order to adjust the glucose concentration. To evaluate oxygen sensitivity, we prepare various partial pressure of oxygen (30, 90, 200 mmHg) with a blood glucose sample (21, 111, 415 mg/dL). The concentration of

glucose was checked using a standard glucose analyzer (GA-1150, Arkray Inc., Japan) and partial pressure of oxygen (mmHg) was checked using a blood gas analyzer (SG-1120, Arkray Inc., Japan)

Electrode preparation

The schematic layout of our SPCE is shown in **Figure 1**. The same volume of a mediator solution containing 200 mM ACES (pH 7.0), 0.1% CHAPS and 60 mM hexaammineruthenium (III) chloride as the sample volume was poured on the sensor dipping zone of the SPCE, which was dried at 30 °C for 30 sec so that the mediator would be deposited on the SPCE. A FADGDH (10 kU/ml) solution containing 0.1% CHAPS was poured (2 U) onto the SPCE, which was dried at 30 °C for 30 sec to deposit the enzyme. The waiting time between the two separate steps is necessary for the prevention of the overflow of reagent due to the limited space at the dipping zone. A picture of the reagents deposited onto the SPCE is shown in **Figure 2**. The sample volume of the sensor can be altered by changing the height of the double-faced tape to 75 µm for the 300-nL sample or 40 µm for the 150-nL sample.

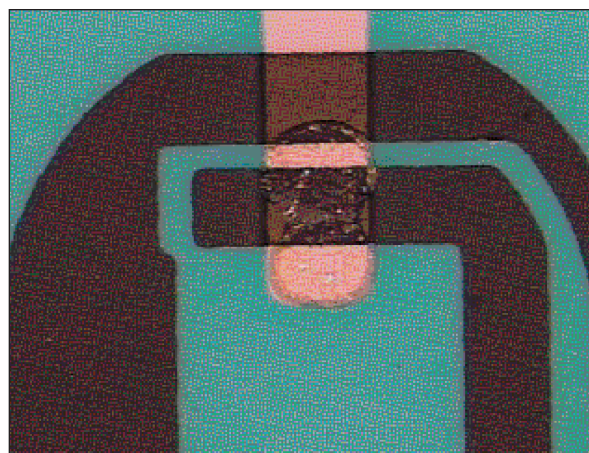


Figure 2: Picture of a reagent-deposited SPCE

A picture of a reagent-deposited SPCE is shown. The upper SPCE is the counter electrode and lower SPCE is the working electrode. The reagent is mainly deposited on the working electrode. The green print is the insulation, which defines the reagent dipping zone.

Electrochemical measurement

All electrochemical measurements were performed using a BAS Voltammetric Analyzer (Bioanalytical Systems, Inc., USA). We used cyclic voltammetry (CV; scan rate: 10 mV/S) to evaluate the FADGDH/Ru-complex-deposited SPCE. Potential vs. the counter-electrode was applied. A glucose solution (100 mg/dL) or distilled water was injected into the sensor and the cyclic voltammogram was monitored.

We used a single-potential time base (TB) to measure the glucose in the blood samples. A potential of +80, 200 or 250 mV vs. the counter electrode was applied immediately when the capillary became filled with the sample. Calibration plots were obtained by averaging the results given by 20 sensors. All measurements were carried out at 25°C.

To evaluate the thermal stability, the sensors were kept in glass bottles with silica gel so as to keep the sensors dry, and the effect of temperature on stability was subsequently investigated. The glass bottles containing the sensors were kept at either 5, 25, 30, 40, 50 or 70°C. After 7, 14, 30 or 60 days of incubation, the sensors were used to measure the glucose in whole blood samples with a glucose concentration of 500 mg/dL. The stability was estimated from the bias from the results given by sensors kept at 5°C.

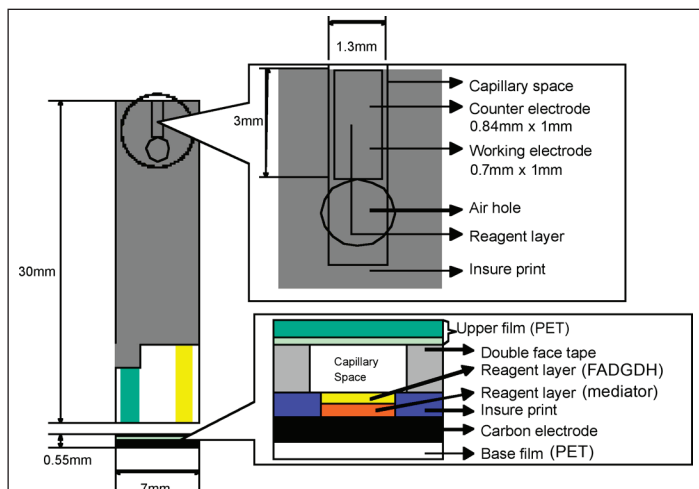


Figure 1: Schematic diagram of the sensor

A schematic diagram of the finished sensor is shown on the left. A close-up of the electrode space and capillary space is shown on the right.

Results and Discussion

Construction of the screen-printed enzyme electrode

Considering that the current requirement in the SMBG market is cost-efficiency,¹⁹ we designed a new SPCE to meet our concept of rapid glucose sensing, using a small sample volume and the novel FADGDH complex. Screen-printed carbon electrodes can be mass-produced and are simple to use. The high catalytic activity of the enzyme can reduce the quantity of enzyme which needs to be deposited onto the SPCE, thereby contributing to the reduction of the cost for the enzyme as well as the reduction of the sample volume; the procedure of taking samples from patients is also much less invasive.

The schematic layout of our SPCE is shown in **Figure 1**. The first and second electrodes were formed on a substrate by screen printing with a carbon paste. Then, insulation printing was performed in order to form the reagent dipping zone. Double-faced tape and base film were used to create a capillary for transporting the sample to the sensor and to the electrode. The size of the working electrode was 0.7 mm x 1 mm, and that of the counter electrode was 0.84 mm x 1 mm; the diameter of the carbon was 10 μm . The sample volume of the sensor was set at 150 nL (LWH: 3 mm x 1.3 mm x 40 μm) or 300 nL (LWH: 3 mm x 1.3 mm x 75 μm), by changing the thickness of double-faced tape.

The electron mediator, hexaammineruthenium (III) chloride, was deposited onto the sensor dipping zone. The FADGDH was also deposited onto the same zone to construct a FADGDH/Ru-complex-deposited SPCE. A picture of the reagent-deposited SPCE is shown in **Figure 2**.

Sensor operation

Figure 3 shows the cyclic voltammogram of the FADGDH/Ru-complex-deposited SPCE, in the presence and absence of glucose. The use of distilled water as the sample did not change the voltammogram (gray line). However, the use of a glucose solution as the sample resulted in a significant change in the voltammogram (solid line). A significant increase in the peak current, I_p , at the peak potential, E_p (80 mV), was observed. From these results, it is reasonable to assume that the FADGDH/Ru-complex-deposited SPCE can be operated at about 80 mV for monitoring glucose solution. The E_p value of 80 mV was much lower than that utilized for potassium ferricyanide as the electron mediator, which is usually about $E_p=150$ mV operating in the glucose solution

(data not shown). The measurement of blood glucose often suffers from the presence of electrochemically active compounds in the sample. A lower applied potential is preferable, to reduce the effect of such electrochemically active ingredients. The waveform and I_p of the FADGDH/Ru-complex-deposited SPCE suggests that the redox cycle of the Ru complex occurred rapidly enough on the electrodes, which enabled a short measurement time. Therefore, the performance of the Ru complex as the electron mediator in this sensor system was evaluated as worthy of further investigation.

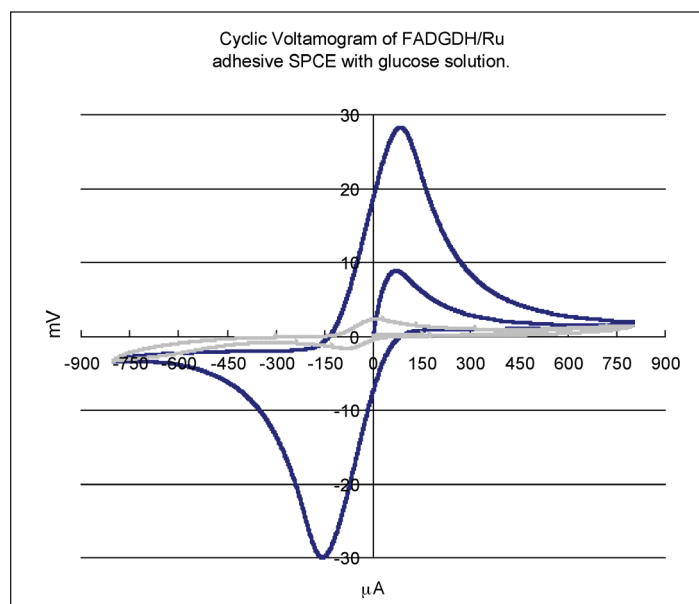


Figure 3: Cyclic voltammogram for the FADGDH/Ru-deposited SPCE Examples of cyclic voltammograms for the FADGDH/Ru-deposited SPCE sensor. The wave form described by the gray line corresponds to the absence of glucose, and the wave form described by the blue line corresponds to 100mg/dL glucose solution. The parameters of measurement are given below. Init P/N = P, V (mV/sec) = 10, Sample Interval (mV) = 1, Quiet Time (sec) = 2, Sensitivity (A/V) = 1E-5, Temperature =25°C.

In order to ensure the correct measurement of glucose in whole blood, the enzyme sensor response was analyzed by the chronoamperometric method. The single-potential TB was obtained from the whole blood samples. **Figure 4** shows the effect of the applied potential on sensor operation toward the sample with 500 mg/dl glucose. The higher applied potential (200 mV and 250 mV) resulted in the higher current compared with those applied 80 mV, which is favorable of the accurate and reproducible measurement. Considering that the achievement of short period of assay is strongly dependent on the rapid and higher sensor signal response, we chose sensor operation at 200 mV.

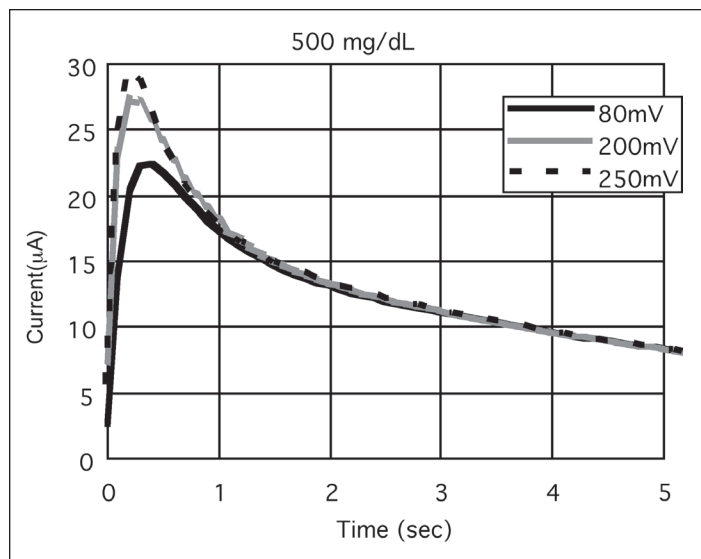


Figure 4: Effect of applied potential of the sensor with blood sample with 500mg/dL glucose.
The single-potential TB for the measurement of 500mg/dL glucose in a blood sample using the FADGDH/Ru-deposited SPCE sensor. A potential of +80 or 200 or 250mV vs. the counter electrode was applied for 5 sec. The hematocrit was adjusted to 42%. The measurements were carried out at 25°C.

Figure 5 show the experiments with different glucose concentration operating at 200 mV. The current peak was reached in about 0.3 sec and then the current gradually decreased. The current at each time point was in correlation with the glucose concentration of the measured sample. Figure 6 shows the correlation between the glucose concentration and the sensor current observed at 1, 2 and 3 seconds. A good linear correlation was observed between the glucose concentration and the current at each time point of measurement within the glucose concentration range tested in this study, i.e., 0-533 mg/dL. These results indicate that, using our newly constructed FADGDH/Ru-complex-deposited SPCE, the blood glucose concentration was correctly determined within 3 sec, even 1 sec. We also investigated the effect of the sample volume on the sensor response. Figure 7 shows the correlation between the glucose concentration and the sensor current observed at 1, 2 and 3 sec after the potential application, but with a 300-nL sample of whole blood. Almost identical correlations were observed as those obtained in the experiments employing a 150-nL sample volume. This result convinced us that a sample volume of 150 nL is sufficient for our sensor to function well. Figure 8 shows the effect of dissolved oxygen concentration (30, 90 and 200 mmHg) on the sensor response toward the sample with 21, 111 or 415 mg/dL glucose. As the enzyme, FADGDH does not utilize oxygen as the electron acceptor, the sensor

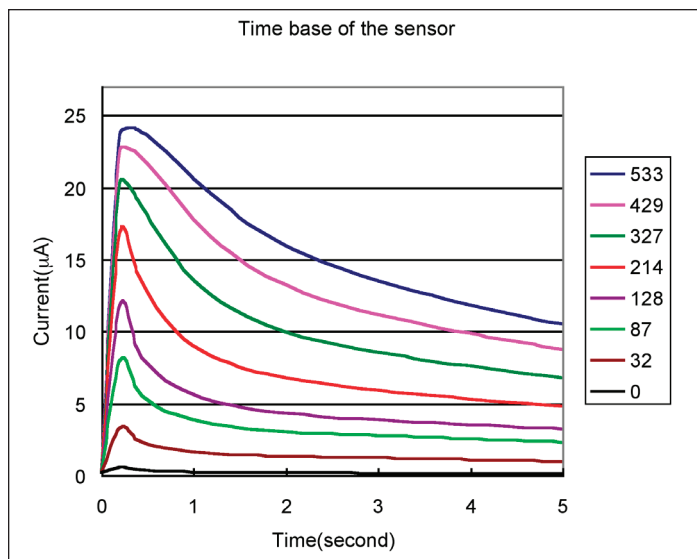


Figure 5: Time base of the sensor
The single-potential TB for the measurement of glucose in a blood sample using the FADGDH/Ru-deposited SPCE sensor. A potential of +200 mV vs. the counter electrode was applied for 5 sec. The samples contained whole blood glucose in concentrations of 0, 32, 86, 127, 213, 327, 429 or 533 mg/dL, and the hematocrit was adjusted to 42%. The measurements were carried out at 25°C.

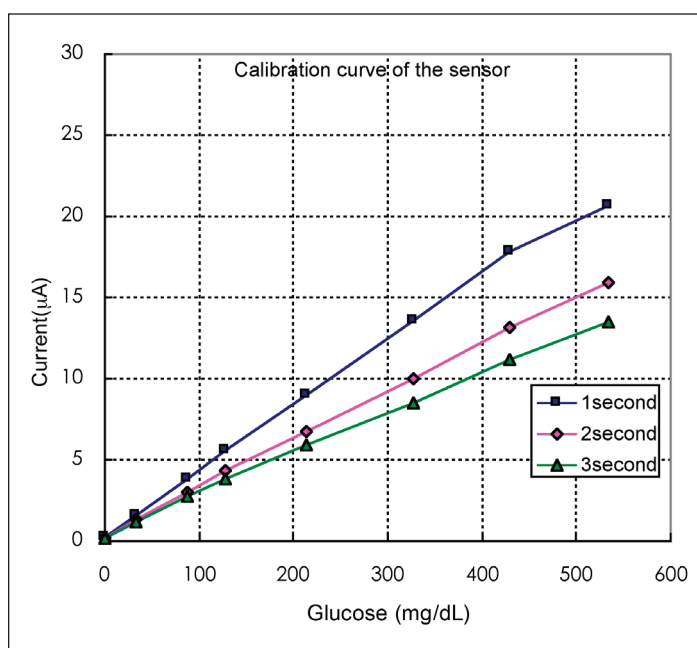


Figure 6: Calibration curves for the sensor with a sample volume of 150 nL
The calibration curves for the finished sensor, for whole blood glucose and various sampling times. The sample volume was set to 150 nL. The glucose concentration was 0, 32, 86, 127, 213, 327, 429 or 533 mg/dL, and the hematocrit was adjusted to 42%. The measurements were carried out at 25°C.

signals were not affected by the change in dissolved oxygen concentration. Therefore, the sensor employed FADGDH is insensitive toward the oxygen.

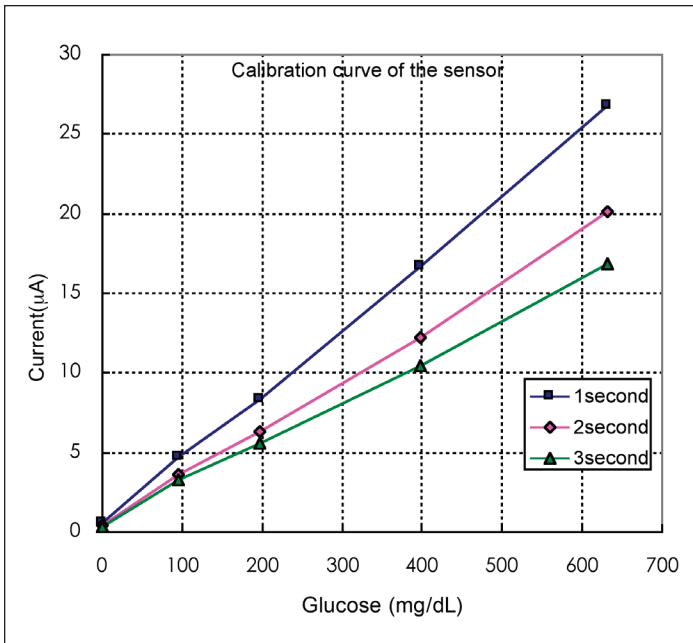


Figure 7: Calibration curves for the sensor with a sample volume of 300 nL
The calibration curves for the finished sensor, for whole blood glucose and various sampling times. The sample volume was set to 300 nL. The glucose concentration was 0, 96, 197, 398 or 632 mg/dL, and the hematocrit was adjusted to 42%. The measurements were carried out at 25°C.

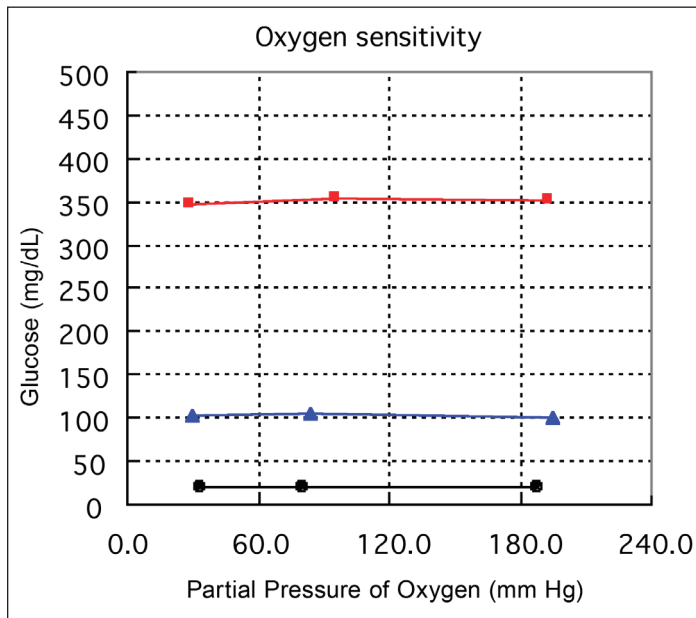


Figure 8: Evaluation of oxygen sensitivity.
The oxygen sensitivity was checked with three glucose concentrations of 21, 111, 415 mg/dL. The partial pressure of oxygen was adjusted to 30, 90, 200 mm Hg for each glucose concentration. The hematocrit was adjusted to 42%. The measurements were carried out at 25°C.

Evaluation of sensor performance

Next, we evaluated the correlation coefficient of the sensor using a 150-nL sample, varying the measurement time. We used 20 sensors for each concentration of whole blood glucose described above (n=160). Our sensors indicated a good correlation: $r=0.995$ for 1 sec of measurement, $r=0.999$ for 2 sec of measurement and $r=0.999$ for 3 sec of measurement (Figures 9a, 9b, and 9c). The longer the measurement time, the higher the precision. However, even with a 1-sec measurement, a high correlation coefficient of 0.995 was achieved.

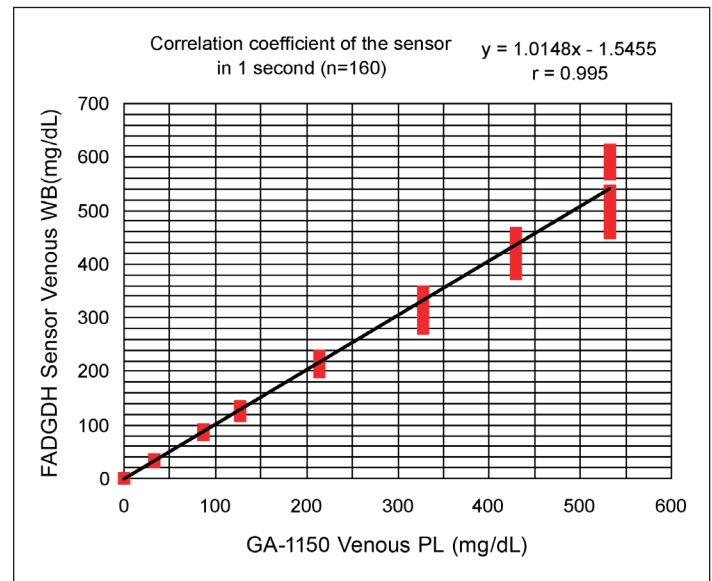


Figure 9a

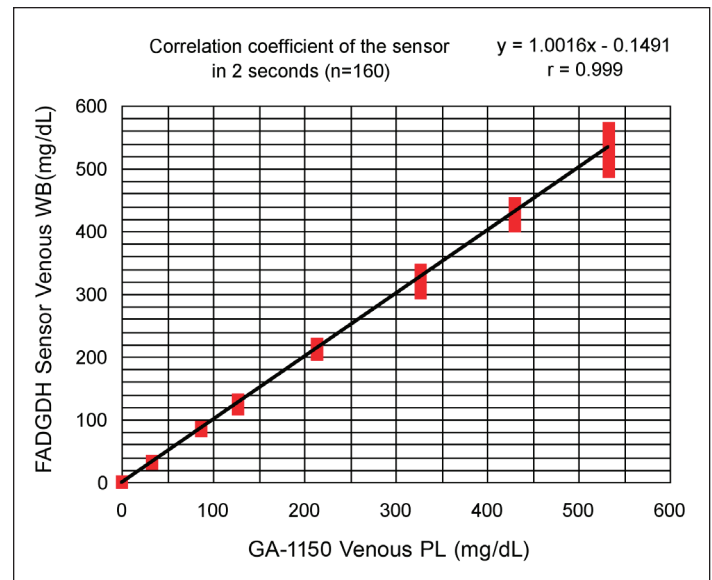


Figure 9b

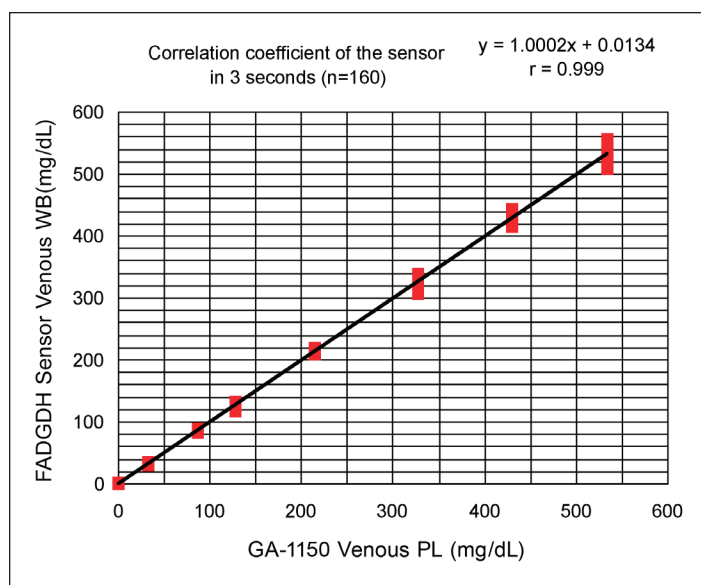


Figure 9c

Figures 9a, 9b, and 9c show the correlation between the measurement results given by the FADGDH/Ru-deposited SPCE sensor and those given by a standard glucose measurement system (Glucose AUTO & STAT GA-1150; ARKRAY) for various measurement times: 1 sec (Figure 9a), 2 sec (Figure 9b), and 3 sec (Figure 9c). The glucose concentration was 0, 32, 86, 127, 213, 327, 429 or 533 mg/dL, and the hematocrit was adjusted to 42%. The measurements were carried out at 25°C. The measurement was repeated 20 times for each glucose concentration (n=160).

Figure 10 shows the reproducibility of measurement, and the CV, for each measurement time. All measurement times achieved good reproducibility; the 1-sec measurement had a CV value of 2.3-4.4%, the 2-sec measurement a CV value of 1.5-3.3% and the 3-sec measurement a CV value of 1.4-2.6%.

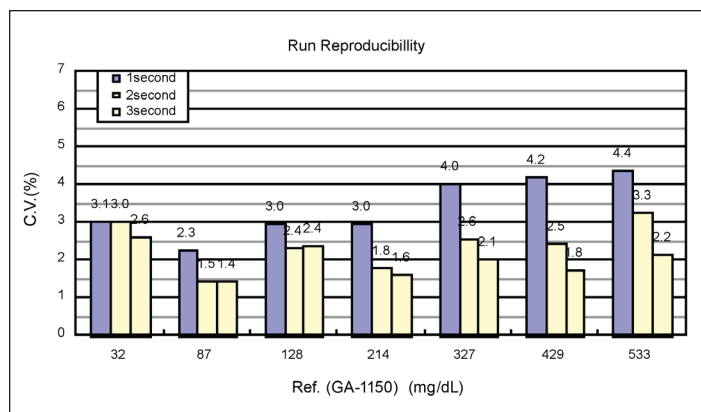


Figure 10: Run reproducibility of the sensor

The reproducibility (n=20) of the measurement results given by the FADGDH/Ru deposited SPCE sensor at each glucose concentration level for various measurement times. The glucose concentration was 0, 32, 86, 127, 213, 327, 429 or 533 mg/dL, and the hematocrit was adjusted to 42%. The measurements were carried out at 25°C.

The standard for CV) described in the ISO15179 "7.2.4" and the R described in the ISO15179 "7.3.3.3" are CV < 4.5% and R > 0.95, respectively. Although our evaluations did not meet the requirement for ISO due to the lack in the number of human subjects, the sensor performance of 1-sec measurement showing R > 0.995 and the CV < 4.4%, within the glucose concentration range of 0-533 mg/dL, indicate the high accuracy of the sensor.

For samples with a lower hematocrit values, the sensor resulted in a higher current because of the presence of many molecules of glucose compared with those with higher hematocrit samples, which is a well known effect in the sensor utilized for SMBG. Since our sensor system is employed with a very small sample volume with a rapid assay, the effect of the hamatocrit affects the reproducibility of the measurement at higher hematocrit values. For example, for a 1 sec sample measurement, the CV is 1.7%, 2.6% and 4.6% for a hematocrit of 20%, 42% and 60%, respectively, whereas for a 2 sec sample measurement, the CV is 1.8%, 1.7% and 3.8% for a hemocrit of 20%, 42% and 60%, respectively. This observation that the assay with shorter period resulted in higher effect of hematocrit at a higher value, may be mainly due to the difficulty in the rapid solubilization of mediator and enzyme on SPCE at the smaller volume of fluid as the consequence of high hematocrit value. Therefore, in order to reduce the effect of hematocrit in the short time assay system, the improvement of solubility of the mediator and enzyme or decrease in the quantity of mediator and enzyme on the SPEC will be essential.

Finally, we evaluated the thermal stability of the thus constructed sensor at incubation temperatures of 25-70°C. No significant decrease in reliability was observed during the 60 days of testing at those temperatures. The sensor was stable even after being incubated at 70°C for 60 days, if it was put in a glass bottle with silica gel (Figure 11). This specification is apparently due to the high thermal stability of the FADGDH.

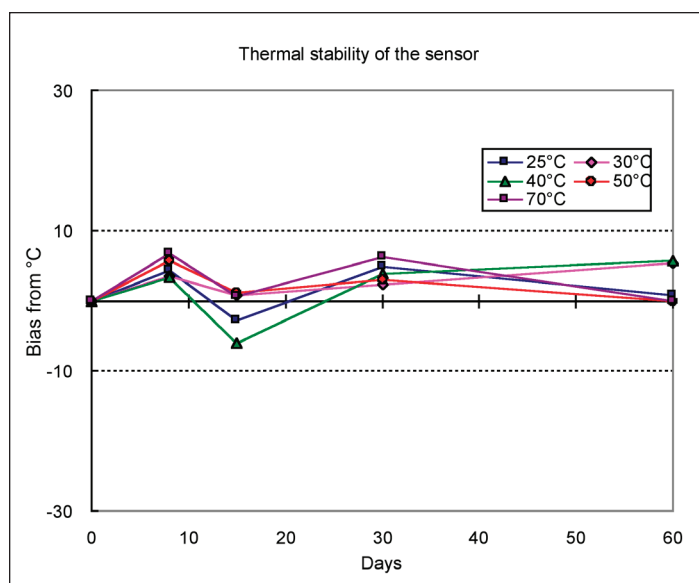


Figure 11: Thermal stability of the sensor

The effect of temperature on stability was investigated. Glass bottles containing sensors were kept at either 5, 25, 30, 40, 50 or 70°C. After 7, 14, 30 or 60 days of incubation, the sensors were used to measure the glucose in whole blood samples with a glucose concentration of 500 mg/dL and the hematocrit adjusted to 42%. The measurements were carried out at 25 °C. The stability was estimated from the bias from the results given by sensors kept at 5 °C.

Conclusion

The main remarkable result of the present research is that the novel thermostable glucose dehydrogenase, FADGDH, has the potential to be used as a component of disposable blood glucose sensors for SMBG. Experiments with an enzyme glucose sensor composed of SPCE and FADGDH-Ru complex revealed that the blood glucose can be determined in 1 sec using a sample volume of 150 nL with high precision and reproducibility. Moreover, the specifications were retained even after the sensor was incubated for 60 days at 70° C, proving that the sensor has high thermal stability, which promises a long shelf life. The sample volume of 150 nL and the measurement time of 1 sec are the highest specifications in the world for currently available commercial glucose sensors designed for SMBG. Disposable glucose sensors using FADGDH/Ru-complex/SPCE are cost-effective and precise enough for use in SMBG. We conclude that our FADGDH/Ru-complex/SPCE-based glucose sensor has a great advantage over the commercial blood glucose sensors currently available in the market.

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