(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 April 2019 (18.04.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/075471 A2

(51) International Patent Classification:
C12Q 1/00 (2006.01)

(21) International Application Number:
PCT/US2018/055904

(22) International Filing Date:
15 October 2018 (15.10.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/572,298 13 October 2017 (13.10.2017) US

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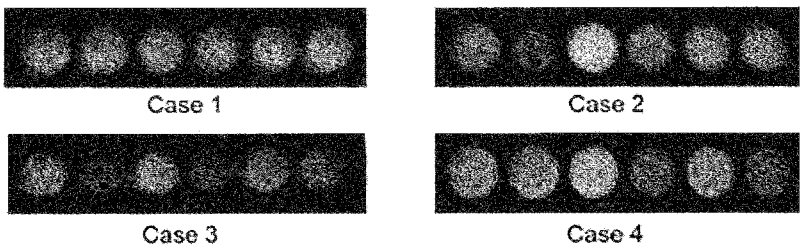
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

(54) **Title:** BIOSENSOR DETECTING GLUCOSE LEVELS OF FREE GLUCOSE, HEMOGLOBIN A1C AND GLYCATED BLOOD PROTEINS IN A SINGLE BLOOD SAMPLE

FIG. 17.



(57) **Abstract:** A biosensor for detecting a glucose level of free glucose, hemoglobin Ale (HbAlc), and glycated blood protein from a single blood sample is disclosed, which includes: a first biosensor for detecting a glucose level of the free glucose, a second biosensor for detecting a glucose level of the HbAlc, a third biosensor for detecting a glucose level of the glycated blood proteins; a fourth biosensor for detecting a hemoglobin level; and a fifth biosensor for detecting non- glycated blood proteins level, where the free glucose, the hemoglobin, the HbAlc, the glycated blood proteins and the non-glycated blood proteins are derived from the single blood sample, where each of the first, second, third and fourth biosensor respectively includes a substrate having a first non-fluorescent dye capable of being converted into a first fluorescent dye by a reaction with the first non-fluorescent dye and H2O2, and where the fifth biosensor includes a substrate having a second non-fluorescent dye capable of being converted into a second fluorescent dye by a reaction with the second non-fluorescent dye and a primary amine in the non-glycated blood proteins.

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BIOSENSOR DETECTING GLUCOSE LEVELS OF FREE GLUCOSE, HEMOGLOBIN A1C AND GLYCATED BLOOD PROTEINS IN A SINGLE BLOOD SAMPLE

This application claims the benefits of U.S. Provisional Application No. 62/572,298, filed October 13, 2017, which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

The present disclosure relates to a biosensor and a method for detecting a glucose level of free glucose, hemoglobin A1c (HbA1c), and glycated blood protein from a single blood sample.

BACKGROUND ART

It is important to maintain the appropriate concentration of glucose in the blood because it is the main energy source for cells as well as the brain. However, if too much glucose formed from the food we eat stays in the blood without interacting smoothly with insulin, generated by the pancreas, the excess glucose can cause diabetes, a serious health problem. Diabetes can cause damage to the eyes, kidneys, and nerves as well as heart diseases. In 2017, Centers for Disease Control and Prevention reported 30.3 million people have diabetes and 84. 1 million adults have pre-diabetes in the U.S. (www.cdc.gov/diabetes/data/statistics/statistics-report.html).

Currently, it is widely believed that Type 2 diabetes can be diagnosed and managed by monitoring the glucose in whole blood or plasma using commercially available glucose meters. However, it is difficult to diagnose and manage diabetes with acceptable accuracy because the variation of the blood sugar quantified before and after consuming food is too wide. In order to diagnose and monitor diabetes more accurately, glycated hemoglobin (or hemoglobin A1c, HbA1c) and glycated blood protein (e.g., glycated albumin) in whole blood are analyzed. This is because the analyses of HbA1c and glycated blood protein can check the average blood glucose levels during a certain period (e.g., HbA1c: 2 ~ 3 months, glycated albumin: 4~ 6 weeks). However, the conventional analytical methods that require complicated instruments such as the HPCL with mass spectrometry are time-consuming and expensive.

On the other hand, it has been believed that commercially available glucose meters, which take 5 sec to measure, are used to quantify just free glucose in whole blood and plasma.

However, the present inventors tested and found that commercially available glucose meters neither accurately detect the concentration of the free glucose, the glucose level of HbA1c or the glucose level of the glycated blood proteins. As shown in Fig. 15, the concentration of glucose in whole blood and plasma were determined with the same glucose meter. However, the glucose meter indicates that glucose concentration in plasma (Fig. 15B) is higher than that in whole blood (Fig. 15A). Also, the concentration of glucose in aqueous solution (Fig 15C), measured after removing blood proteins and red blood cells using a 10K filter, was the highest. Additionally, glycated blood proteins (Fig. 15D) as well as HbA1c (Fig. 15E) can be quantified with the glucose meter in the absence of glucose. Fig. 15 shows that the glucose concentration (mg/dL) of Fig 15A comes from the mixture of the free glucose, glycated blood proteins, and HbA1c in whole blood, whereas that of Fig 15B was determined by the sum of the free glucose and glycated blood proteins in plasma. Thus, the total glucose concentration in whole blood should be higher than that in plasma. However, the former was lower than the latter because whole blood is more complicated and contains more interferences than plasma. As evidence, the concentration of glucose measured in aqueous solution containing just free glucose (Fig. 15C) was higher than that of Figs. 15A and 15B.

Although the present inventors found that the commercially available glucose meter can quantify glycated blood proteins and HbA1c, it cannot be applied to diagnose diabetes accurately and effectively. This is because the commercially available glucose meters cannot quantify total blood proteins and hemoglobin to determine the percentile concentration of glycated blood proteins and HbA1c.

Therefore, a method of accurately detecting the glucose level of free glucose, hemoglobin A1c (HbA1c), and glycated blood protein from a single blood sample has yet to be developed.

SUMMARY

According to one aspect of the present invention, a biosensor for detecting a glucose level of free glucose, hemoglobin A1c (HbA1c), and glycated blood protein from a single blood sample is provided, which comprises: a first biosensor for detecting a glucose level of the free glucose, a second biosensor for detecting a glucose level of the HbA1c, a third biosensor for detecting a glucose level of the glycated blood proteins; a fourth biosensor for detecting a hemoglobin level; and a fifth biosensor for detecting non-glycated blood proteins level, wherein

the free glucose, the hemoglobin, the HbA1c, the glycated blood proteins and the non-glycated blood proteins are derived from the single blood sample, wherein each of the first, second, third and fourth biosensor respectively includes a substrate having a first non-fluorescent dye capable of being converted into a first fluorescent dye by a reaction with the first non-fluorescent dye and H_2O_2, and wherein the fifth biosensor includes a substrate having a second non-fluorescent dye capable of being converted into a second fluorescent dye by a reaction with the second non-fluorescent dye and a primary amine in the non-glycated blood proteins.

The first, second, third, fourth and fifth biosensors may include 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) reagent. The first fluorescent dye may be at least one selected from the group consisting of 2-aminobenzoyl (Abz), N-methyl-anthraniloyl (N-Me-Abz), 5-(dimethylamino)naphthalene-1-sulfonyl (Dansyl), 5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid (EDANS), 7-dimethylaminocoumarin-4-acetate (DMACA), 7-amino-4-methylcoumarin (AMC), (7-methoxycoumarin-4-yl)acetyl (MCA), rhodamine, rhodamine 101, rhodamine 110 and resorufin. The first fluorescent dye may be resorufin. The second fluorescent dye may be fluorescamine. The first, second and third biosensors may further comprise a horseradish peroxidase (HRP) and a glucose oxidase (GOx). The 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) reagent may comprise an ODI and H_2O_2.

In accordance with another aspect of the present invention, a kit for detecting a glucose level of free glucose, hemoglobin A1c (HbA1c), and glycated blood protein from a single blood sample is provided, the kit comprises: the above biosensor; and a container. The kit may further comprise a buffer; and 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) reagent.

In yet another aspect of the present invention, a method of detecting a glucose level of a single blood sample is provided, the method comprises: providing a free glucose solution, a glycated blood proteins solution, a hemoglobin A1c (HbA1c) solution, a hemoglobin solution, and a non-glycated blood proteins solution from the single blood sample; separately detecting the glucose levels of the free glucose solution, the glycated blood proteins solution and the HbA1c solution and the level of hemoglobin by using a first enzyme assay with 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) detection; detecting the level of non-glycated blood proteins by using a second enzyme assay with ODI-CL detection; measuring a relative glucose level of HbA1c based on the level of the hemoglobin; and measuring a relative glucose level of glycated

blood proteins based in the level of the non-glycated blood proteins. The first enzyme assay with 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) detection may be performed by using a first non-fluorescent dye capable of being converted into a first fluorescent dye by a reaction with the first non-fluorescent dye and H_2O_2. The second enzyme assay with 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) detection may be performed by using a second non-fluorescent dye capable of being converted into a second fluorescent dye by a reaction with the second non-fluorescent dye and a primary amine in the non-glycated blood proteins. The first fluorescent dye may be resorufin, and the second fluorescent dye nay be fluorescamine. The steps of detecting the glucose levels, detecting the level of non-glycated blood proteins and the level of hemoglobin may be performed for 1-5 minutes at room temperature.

In yet another aspect of the present invention, a method of diagnosing type 2 diabetes is provided, which comprises: detecting a glucose level of a single blood sample from a patient using the above method, and comparing the glucose level of the patient with a glucose level of a blood sample from a third party.

These and other aspects will be appreciated by one of ordinary skill in the art upon reading and understanding the following specification.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and advantages of the present invention will become more apparent to those of ordinary skill in the art by describing exemplary embodiments thereof in detail with reference to the accompanying drawings, in which:

Fig. 1 shows the reaction mechanism for the quantification of free glucose using enzyme assay with ODI-CL detection. R_1 and R_2 are H, CH_3, or CH_2CH_3.

Fig. 2 is a photo showing the effect of free glucose concentration in the reactions (1) and (2) of Fig. 1 for 4 min at room temperature. (The concentration (mM) of free glucose from left: 0, 0.063, 0.13, 0.25, 0.5, 1, 2, and 4. [GOx] = 0.45 U/ml, [Amplex Red] = 1.6 μM, [HRP] = 50 mU/ml.)

Fig. 3 is a graph showing the effect of incubation time in the reactions (1) and (2) of Fig. 1 at room temperature. ([glucose] = 1 mM, [GOx] = 0.5 U/ml, [HRP] = 50 mU/ml, [Amplex Red] = 3.2 μM.)

Fig. 4 is a diagram showing the role of hemoglobin in red blood cell.

Fig. 5 shows a reaction mechanism for the quantification of hemoglobin in a sample.

Fig. 6 is graphs showing the effect of variables in the mimicking enzyme reaction using hemoglobin. [(A) Effect of pH, (B) Effect of Tris-HCl buffer concentration, (C) Effect of H_2O_2, (D) Effect of Amplex Red concentration].

Fig. 7 depicts the structure of HbA1c.

Fig. 8 shows the reaction mechanism for enzyme assay to quantify HbA1c in a sample.

Fig. 9 is a graph showing the relative CL intensity measured in enzyme assay with ODI-CL detection in the absence and presence of HbA1c. ([GOx] = 0.5 U/ml, [HRP] = 50 mU/ml, [Amplex Red] = 3.2 μM.)

Fig. 10 shows the reaction mechanism of enzyme assay for the analysis of glycated blood proteins.

Fig. 11 is a photo showing formation of resorufin from the enzyme reaction in the presence of glycated blood proteins. (The concentration (mg/ml) of glycated blood proteins from the left: 3.1, 6.3, 12.5, 25, 50, 100.)

Fig. 12 shows the reaction mechanism of fluorescamine and primary amine to produce fluorescent dye.

Fig. 13 shows the reaction mechanism for the quantification of non-glycated proteins in a sample. (R is the non-glycated protein.)

Fig. 14 is a graph showing the ratio of relative CL intensities measured in the absence (CL_0) and presence of non-glycated protein (CL) with ODI-CL detection.

Fig. 15 is photos showing the measurement of triple biomarkers in a human sample. [(A) Whole blood, (B) Plasma, (C) Free glucose in aqueous solution after removing plasma and red blood cells, (D) Glycated proteins diluted in PBS (pH 7.4) after removing free glucose and red blood cells, (E) HbA1c in red blood cells.]

Fig. 16 is graphs showing the calibration curves capable of rapidly quantifying free glucose (A), glycated blood protein (B), non-glycated blood protein (C), HbA1c (D), and total hemoglobin (E) in a sample. (Incubation time at room temperature was 3 min.)

Fig. 17 is photos showing the real-time analyses of the triple biomarkers, non-glycated proteins, and total hemoglobin. (From left (1) whole blood, (2) Free glucose in aqueous solution

after removing plasma and red blood cells, (3) non-glycated proteins (4) glycated proteins (5) total hemoglobin and (6) HbA1c.)

Fig. 18 is graphs showing (A) Measurement of individual free glucose & glycated blood proteins extracted from plasma and the mixture in plasma. ([GOx] = 0.5 U/ml, [HRP] = 0.4 U/ml, [Amplex Red] = 14 μM), and (B) Measurement of hemoglobin only and hemoglobin mixed with glucose and/or HbA1c. ([Hb] = 1.4 mg/ml, [glucose] = 20 μM, [HbA1c] = 12.5 μg/ml, [Amplex Red] = 7 μM, and [GOx] = 0.45 U/ml.)

DETAILED DESCRIPTION

The concentration of glucose in blood or plasma can be determined with the enzyme assay using glucose oxidase (GOx), horseradish peroxidase (HRP) and an appropriate substrate for electrochemical, colorimetric, fluorescence, or chemiluminescence detection. Using the enzyme assay, it is possible to rapidly determine high concentrations of glucose existing in blood.

Enzyme immunoassay with 1,1'-oxalydiimidazole chemiluminescence (ODI-CL) detection can be more sensitive than those with other optical and electrochemical detections. Thus, according to an embodiment of the present invention, the blood glucose can be rapidly analyzed with the enzyme assay with ODI-CL detection.

HbA1c is formed with the non-enzymatic attachment of glucose to hemoglobin. The concentration of HbA1c accumulated in red blood cells is dependent on the blood glucose level. Glycated blood protein is also formed from the non-enzymatic attachment of glucose to blood protein. Based on the mechanism of HbA1c and glycated protein formation, the present inventors found that it is possible to develop modified enzyme assays with ODI-CL detection capable of rapidly quantifying and monitoring HbA1c and glycated blood protein in whole blood due to the similarity of chemical and physical properties of glucose bound to hemoglobin or blood protein, and developed a biosensor and a method which consecutively quantify or simultaneously monitor/detect the triple biomarkers (e.g., free glucose, HbA1c, glycated blood protein) using three different enzyme assays with ODI-CL detection. According to an embodiment of the present invention, it is also possible to detect the glucose level of the triple

markers in a single blood sample at any time of day for accurate diagnosis and precise management of diabetes.

The biosensor may use 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) detection and can consecutively or simultaneously detect triple biomarkers (e.g., free glucose, hemoglobin A1c (HbA1c), glycated blood proteins), which may be used for the diagnosis of diabetes. The inventors of the present invention found that the glucose of HbA1c and the glucose of glycated blood proteins have the same chemical properties as free glucose because the enzyme reaction between HbA1c (or glycated blood protein) and glucose oxidase (GOx) is as rapid as that between free glucose and GOx. Thus, H_2O_2 is rapidly formed from the enzyme reaction occurring in each well containing GOx and one of the triple biomarkers. H_2O_2 formed from the enzyme reaction also may consecutively react with a substrate having a non-fluorescent dye, *e.g.*, Amplex Red (non-luminescent substrate), to produce resorufin, luminescent dye, in the well during a short period (e.g., 3-min) of incubation at room temperature. Resorufin formed in the each well emit lights when H_2O_2 and ODI were added in the well. The strength of the light emitted in the well was proportionally enhanced with the increase of biomarker concentration. Thus, it is expected that the present invention may provide a cost-effective and easy-to-use diagnostic method, which can accurately and precisely detect the glucose level of the blood sample, thereby diagnose diabetes at any time of day, regardless of before or after food intake.

The biosensor according to an embodiment of the present invention includes a first biosensor for detecting a glucose level of the free glucose, a second biosensor for detecting a glucose level of the HbA1c, a third biosensor for detecting a glucose level of the glycated blood proteins. Each of the first, second, third and fourth biosensor respectively includes a substrate having a first non-fluorescent dye capable of being converted into a first fluorescent dye by a reaction with the first non-fluorescent dye and H_2O_2.

The first fluorescent dye used in the fluorogenic substrate may be at least one of 2-aminobenzoyl (Abz), N-methyl-anthraniloyl (N-Me-Abz), 5-(dimethylamino)naphthalene-1-sulfonyl (Dansyl), 5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid (EDANS), 7-dimethylaminocoumarin-4-acetate (DMACA), 7-amino-4-methylcoumarin (AMC), (7-methoxycoumarin-4-yl)acetyl (MCA), rhodamine, rhodamine 101, rhodamine 110 and resorufin. In this specification, resorufin is used as an example, but other fluorescent dye can be used alone or in combination with each other.

Whole blood can be separated into plasma and red blood cells. Plasma can be also separated to blood proteins and free glucose aqueous solution. Blood proteins include glycated blood proteins and non-glycated blood proteins. Also, red blood cells include hemoglobin and HbA1c. Thus, free glucose, hemoglobin, HbA1c, glycated blood proteins and non-glycated blood proteins can be derived from a single blood sample.

The present invention also detect the level of hemoglobin in red blood cells, which may be detected with a modified enzyme assay with ODI-CL detection. Hemoglobin can be used as mimicking HRR instead of HRP during the ODI-CL detection.

The present invention also detect the level of the non-glycated proteins in the blood sample. Because the non-glycated proteins do not include a glucose, a non-fluorescent dye, (capable of being converted into a fluorescent dye by a reaction with the non-fluorescent dye and a primary amine in the non-glycated blood proteins,) *e.g.,* fluorescamine, can be used. The fluorescamine may be dissolved in acetone and mixed with non-glycated protein in phosphate buffer (pH 8.5), and then, incubated for a short period (e.g., 3 min) at room temperature to produce fluorescent dye. The concentration of fluorescamine formed after the 3-min reaction was dependent on the concentration of non-glycated proteins in a sample. Thus, the strength of the light emission from fluorescent dye in the presence of ODI-CL reagents was enhanced with the increase of non-glycated blood proteins in a sample. A luminometer may be used to measure the strength of the light, the relative CL intensity, emitted in a sample.

The detection of the glucose levels, detection of the level of non-glycated blood proteins and the level of hemoglobin may be performed for 1-5 minutes at room temperature, more preferably, 3-4 minutes.

A biosensor as described above may be provided in the form of a kit. In one embodiment of the present invention, the kit includes the above-described biosensor and a container. The kit may further include a buffer and an ODI-CL reagent (e.g., ODI and H_2O_2).

The above method can be applied for diagnosing type 2 diabetes by detecting a glucose level of a single blood sample from a patient, and comparing the glucose level of the patient with a glucose level of a blood sample from a third party.

Hereinafter, embodiments will be explained in detail to particularly explain the present invention. The present invention may, however, be embodied in different forms and should not

be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the inventive concept to those skilled in the art.

Detection of the free glucose level

As shown in Fig. 1, free glucose in a sample is rapidly quantified using the enzyme assay with, *e.g.,* ODI-CL detection. First, H_2O_2 is formed from the reaction of glucose and glucose oxidase. Then, resorufin, which is a fluorescent dye, is formed from the reaction between H_2O_2 and Amplex Red, which is non-fluorescent substrate, in the presence of horseradish peroxidase (HRP). The color of resorufin in Tris-HCl buffer (pH 8) is pink, whereas Amplex Red is a colorless substrate. The yields of H_2O_2 and resorufin formed from the reactions (1) and (2) of Fig. 1 are dependent on the concentration of glucose in a sample and the reaction time in a well containing glucose, glucose oxidase, and Amplex Red. Fig. 2 shows that the strength of the pink color indicating the formation of resorufin in the well gets proportionally stronger with the increase of glucose in a sample because the yield of resorufin in the reaction (2) is enhanced with the increase of H_2O_2 formed from the reaction (1), Also, Fig. 2 shows that the reactions (1) and (2) of Fig. 1 occur immediately and consecutively in each well during a certain incubation time (*e.g.*, 4 min) at room temperature.

As shown in the reaction (3) of Fig. 1, high-energy intermediate (X) formed from the reaction between ODI and H_2O_2 transfer energy to resorufin formed from the reactions (1) and (2) based on the principle of chemiluminescence resonance energy transfer (CRET). The resorufin in the ground state goes up to the excited state due to the CRET. Then, resorufin in the excited state goes back to the ground state with the emission of red light as shown in the reaction (3) of Fig. 1.

Fig. 3 shows that relative CL intensity is dependent on the incubation time of the mixture (e.g., glucose, GO, HRP, Amplex Red) in a reaction well. Relative CL intensity was enhanced with the increase of the incubation time.

Detection of the level of hemoglobin

As shown in Fig. 4, hemoglobin (Hb) is the oxygen-transport metalloprotein with Heme group which is an iron-containing compound of the porphyrin class. The concentration of

hemoglobin in human blood varies depending on the age and gender. For example, the range (14 – 17.5 g/dL) and mean (15.7 g/dL) of hemoglobin concentration for adult men are higher than those (12.3 – 15.3 g/dL, 13.8 g/dL) for adult women. Thus, it is important to quantify hemoglobin in a patient sample. This is because it is impossible to determine the percentile concentration of HbA1c without considering the level of the total hemoglobin (for the diagnosis and monitoring of type 2 diabetes).

The Heme group of hemoglobin in red blood cells is known as a mimic enzyme of HRP. The inventors of the present application confirmed that hemoglobin in a sample can act as a mimic enzyme, instead of HRP, in the reaction between Amplex Red and H_2O_2 to form resorufin, as shown in Fig. 5. In other words, it may be possible to rapidly quantify hemoglobin in a sample based on the reaction mechanism shown in Fig. 5.

As shown in Fig. 6(A), the efficiency of the mimicking enzyme assay for the analysis of hemoglobin may be dependent on the pH of the Tris-HCl buffer solution. It was reported that the best pH for CRET between fluorescent dye and X formed in the ODI-CL reaction is 7. However, Fig. 6(A) shows that the best pH for the mimicking enzyme assay may be 8. The results indicate that the appropriate pH for the reaction of Amplex Red and H_2O_2 in the presence of hemoglobin to produce resorufin is 8 or higher. In other words, the results shown in Fig. 6 were determined by the combination of both the mimicking enzyme and ODI-CL reactions. The best pH in the mimicking enzyme assay using hemoglobin was the same as that in a conventional enzyme assay with HRP. In addition, in Fig. 6(B), 10 mM Tris-HCl buffer (pH 8) is used for the analysis of hemoglobin in a sample based on the results of Fig 6(A). Fig. 6(C) shows that the mimicking enzyme assay is not dependent on the concentration of H_2O_2 under the statistically acceptable error range. Thus, 0.4 mM H_2O_2 was used for the analysis of hemoglobin in a sample. As shown in Fig. 6(D), relative CL intensity after a 4-min incubation for the mimicking enzyme assay at room temperature was enhanced with the increase of Amplex Red concentration. However, the relative CL intensity in the presence of 31.12 μM Amplex Red is only 14 % higher than that in the presence of 15.56 mM. Thus, 15 mM Amplex Red was used for the development of a cost-effective biosensor capable of rapidly sensing hemoglobin in a sample in the Example.

Detection of the glucose level of HbA1c

Glucose sticks to the N-terminal of hemoglobin in human blood to make glycated

hemoglobin molecules, called HbA1c. In other words, glucose can bind with hemoglobin through a non-enzymatic attachment. Thus, it is possible that the chemical properties of glucose of HbA1c may be always the same as those of free glucose in a sample as shown in Fig. 7.

Based on the reaction mechanism shown in Fig. 1, an enzyme assay for the quantification of HbA1c are shown in Fig. 8. The enzyme reaction between HbA1c and GOx was as rapid as that between free glucose and GOx. Thus, developing an enzyme assay with ODI-CL detection for the analysis of HbA1c in a sample may be possible based on the reaction mechanism shown in Fig. 8. For example, Fig. 9 shows that relative CL intensity was enhanced with the increase of HbA1c in a sample.

Detection of the glucose level of glycated blood proteins

Glycated blood proteins are formed from the non-enzymatic reaction between glucose in plasma and the primary amine of blood proteins. A modified reaction mechanism for the analysis of glycated blood protein is shown in Fig. 10. First, glycated blood proteins react with GOx to form H_2O_2 capable of reacting with Amplex Red in the presence of HRP. Resorufin formed from the enzyme reaction emits red light with the addition of ODI-CL reagents as shown in Fig. 10.

For example, the strength of the pink color shown in the well was enhanced with the increase of glycated blood protein concentration in the sample as shown in Fig. 11.

Detection of the level of non-glycated blood proteins

In order to quantify non-glycated blood proteins, a new analytical method based on the reaction mechanism shown in Fig. 12 is developed. This is because non-glycated blood proteins do not act as HRP or mimicking HRP enzyme like hemoglobin. As shown in Fig. 12, fluorescamine, a non-fluorescent dye, in acetone reacts rapidly with primary amine in an aqueous buffer to produce fluorescent dye.

A reaction mechanism for the quantification of non-glycated blood proteins in a sample is developed as shown in Fig. 13. Primary amine present at the N-terminal of non-glycated blood proteins in phosphate buffer (pH 8.5) reacts with fluorescamine to produce fluorescent dye. Finally, the fluorescent dye emits bright green light with the addition of ODI-CL reagents. Fig. 14 shows that non-glycated protein bound with fluorescent dye emits bright (green) light.

EXAMPLE

Hereinafter, example and experimental examples will be further explained, however the present invention is not limited to the following examples and experimental examples.

Chemicals and materials

Glucose, glucose oxidase, glycated human serum albumin, human serum albumin, horseradish peroxidase (HRP), and fluorescamine were purchased from Sigma-Aldrich. Hemoglobin, HbA1c was purchased from Lee Biosolutions. Bis(2,4,6-trichloro)phenyl oxalate, and 4-methyl imidazole were purchased from TCI America. Amplex Red was purchased from Cayman Chemical. Deionized water (HPLC grade), isopropyl alcohol (HPLC grade) and ethyl acetate (HPLC grade) were purchased from EMD. 3% and 30% H_2O_2 were purchased from VWR. Single donor human whole blood standards drawn from healthy people in FDA-licensed facilities were purchased from Innovative Research.

Separation of free glucose, blood proteins, and red blood cells in whole blood

Whole blood in a 1.5-ml centrifuge tube was centrifuged at 2000 rpm for 15 min at 4 °C using CS-15R centrifuge (Beckman). After the centrifugation, plasma and red blood cells were obtained. Then, plasma (500 μl) was transferred to the reservoir of centrifugal concentrator (Nanosep, 10K, Pall filtron) capable of separating blood proteins and free glucose. Plasma in the centrifugal concentrator was centrifuged at 8000 rpm for 10 min at 4 °C. After the centrifugation, free glucose in aqueous solution and blood protein were separated. Blood proteins remaining in the reservoir of centrifugal concentration were diluted with PBS buffer (pH 7.4, 300 μl). Fresh free glucose, blood proteins, and red blood cells were prepared every time before conducting the experiment. All the biological samples were prepared in a biosafety cabinet.

Detection of free glucose, glycated blood proteins, non-glycated blood proteins, HbA1c, and total hemoglobin in a single blood sample

Based on the preliminary research results described above, 5 calibration curves for the analyses of free glucose, glycated blood protein, non-glycated blood protein, HbA1c, and total hemoglobin in a sample was obtained as shown in Fig. 16. It is expected that it will be possible to diagnose diabetes accurately and monitor and manage it effectively using the 5 calibration

curves. This is because they can rapidly quantify the triple biomarkers (free glucose, glycated blood protein, HbA1c) non-glycated blood protein, and total hemoglobin in a patient sample with statistically acceptable accuracy, precision, and reproducibility.

Five biosensors (a biosensor for detecting a glucose level of the free glucose (#2), a biosensor for detecting a glucose level of the HbA1c (#6), a biosensor for detecting a glucose level of the glycated blood proteins (#4); a biosensor for detecting a hemoglobin level (#5); and a biosensor for detecting non-glycated blood proteins level(#3)) and additional biosensor for detecting a glucose level of whole blood (#1) were prepared by the above described manners. Four blood samples were separated and applied to the above biosensors, and the intensities of the emitted lights were observed and measured in Fig. 17.

As shown in Fig. 17, the biosensor can simultaneously monitor the triple biomarkers, non-glycated proteins, and total hemoglobin within 3 min at room temperature. The possible evaluation for each of the four cases shown in Fig. 17 is below:

Case 1: Bright light is emitted from all wells. This represents a critical diabetes patient.

Case 2: Dim light is emitted from the second well while bright light is emitted from the rest of the wells. This represents a diabetes patient who has begun controlling free glucose with the help of a medical doctor.

Case 3: Dim light is emitted from the second, forth, and sixth wells, each containing one of the triple biomarkers. The brightness of the two wells containing non-glycated proteins or total hemoglobin is dependent on individual patient sample. This case represents a patient whose concentration of the triple biomarkers has dropped as a result at least 3 months of diabetes treatment.

Case 4: Dim light emitted from the fourth and sixth wells while bright light is emitted from the rest of the wells. This represents a normal person whose concentration of free glucose has increased after a meal.

Based on the reaction mechanisms of Fig. 1 and Fig. 10, relative CL intensities emitted in the presence of free glucose, glycated blood proteins and the mixture were measured as shown in Fig. 18(A). The relative CL intensity measured in plasma containing the mixture of free glucose

and glycated blood proteins is similar to that in the presence of free glucose separated from the plasma. Based on the results, it is confirmed that the strength of CL emission is dependent on the various biomaterials in a sample. Also, Fig. 18(A) indicates that it is impossible to quantify just free glucose in plasma. In other words, the results of Figs. 15 and 18(A) are clear evidences that it is difficult to apply the conventional glucose meters to diagnose diabetes because they cannot quantify just free glucose in plasma containing glycated blood proteins.

Based on the reaction mechanisms of Fig. 1 and Fig. 8, relative CL intensities emitted in the presence of HbA1c in hemoglobin and the mixture containing hemoglobin, free glucose, and additional HbA1c were measured in Fig. 18(B). Even though light emitted from the mixture containing hemoglobin, free glucose, and additional HbA1c was the strongest, it is impossible to selectively determine the concentration of free glucose or HbA1c in a sample containing free glucose and HbA1c.

Furthermore, the results of Figs. 16-18 show that the biosensor of the present invention can be applied to accurately diagnose and precisely monitor diabetes at any time of day, whereas commercially available glucose meters cannot as shown in Fig. 15.

It is to be understood that the above-described biosensor and method are merely illustrative embodiments of the principles of this disclosure, and that other compositions and methods for using them may be devised by one of ordinary skill in the art, without departing from the spirit and scope of the invention. It is also to be understood that the disclosure is directed to embodiments both comprising and consisting of the disclosed parts.

WHAT IS CLAIMED IS:

1. A biosensor for detecting a glucose level of free glucose, hemoglobin A1c (HbA1c), and glycated blood protein from a single blood sample, comprising:

a first biosensor for detecting a glucose level of the free glucose,

a second biosensor for detecting a glucose level of the HbA1c,

a third biosensor for detecting a glucose level of the glycated blood proteins;

a fourth biosensor for detecting a hemoglobin level; and

a fifth biosensor for detecting non-glycated blood proteins level,

wherein the free glucose, the hemoglobin, the HbA1c, the glycated blood proteins and the non-glycated blood proteins are derived from the single blood sample,

wherein each of the first, second, third and fourth biosensor respectively includes a substrate having a first non-fluorescent dye capable of being converted into a first fluorescent dye by a reaction with the first non-fluorescent dye and H_2O_2, and

wherein the fifth biosensor includes a substrate having a second non-fluorescent dye capable of being converted into a second fluorescent dye by a reaction with the second non-fluorescent dye and a primary amine in the non-glycated blood proteins.

2. The biosensor of claim 1, wherein the first, second, third, fourth and fifth biosensors include 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) reagent.

3. The biosensor of claim 1, wherein the first fluorescent dye is at least one selected from the group consisting of 2-aminobenzoyl (Abz), N-methyl-anthraniloyl (N-Me-Abz), 5-(dimethylamino)naphthalene-1-sulfonyl (Dansyl), 5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid (EDANS), 7-dimethylaminocoumarin-4-acetate (DMACA), 7-amino-4-methylcoumarin (AMC), (7-methoxycoumarin-4-yl)acetyl (MCA), rhodamine, rhodamine 101, rhodamine 110 and resorufin.

4. The biosensor of claim 1, wherein the first fluorescent dye is resorufin.

5. The biosensor of claim 1, wherein the second fluorescent dye is fluorescamine.

6. The biosensor of claim 1, wherein the first, second and third biosensors further comprises a horseradish peroxidase (HRP) and a glucose oxidase (GOx).

7. The biosensor of claim 2, wherein the 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) reagent comprises an ODI and H_2O_2.

8. A kit for detecting a glucose level of free glucose, hemoglobin A1c (HbA1c), and glycated blood protein from a single blood sample, the kit comprising:

the biosensor of claim 1; and

a container.

9. The kit of claim 8, further comprising:

a buffer; and

1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) reagent.

10. A method of detecting a glucose level of a single blood sample, comprising:

providing a free glucose solution, a glycated blood proteins solution, a hemoglobin A1c (HbA1c) solution, a hemoglobin solution, and a non-glycated blood proteins solution from the single blood sample;

separately detecting the glucose levels of the free glucose solution, the glycated blood proteins solution and the HbA1c solution and the level of hemoglobin by using a first enzyme assay with 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) detection;

detecting the level of non-glycated blood proteins by using a second enzyme assay with ODI-CL detection;

measuring a relative glucose level of HbA1c based on the level of the hemoglobin; and

measuring a relative glucose level of glycated blood proteins based in the level of the non-glycated blood proteins.

11. The method of claim 10, wherein the first enzyme assay with 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) detection is performed by using a first non-fluorescent dye

capable of being converted into a first fluorescent dye by a reaction with the first non-fluorescent dye and H_2O_2.

12. The method of claim 10, wherein the second enzyme assay with 1,1'-oxalyldiimidazole chemiluminescence (ODI-CL) detection is performed by using a second non-fluorescent dye capable of being converted into a second fluorescent dye by a reaction with the second non-fluorescent dye and a primary amine in the non-glycated blood proteins.

13. The method of claim 11, wherein the first fluorescent dye is resorufin.

14. The method of claim 12, wherein the second fluorescent dye is fluorescamine.

15. The method of claim 10, wherein the steps of detecting the glucose levels, detecting the level of non-glycated blood proteins and the level of hemoglobin are performed for 1-5 minutes at room temperature.

16. A method of diagnosing type 2 diabetes, comprising:

detecting a glucose level of a single blood sample from a patient using the method of claim 8, and

comparing the glucose level of the patient with a glucose level of a blood sample from a third party.

DRAWINGS

FIG. 1

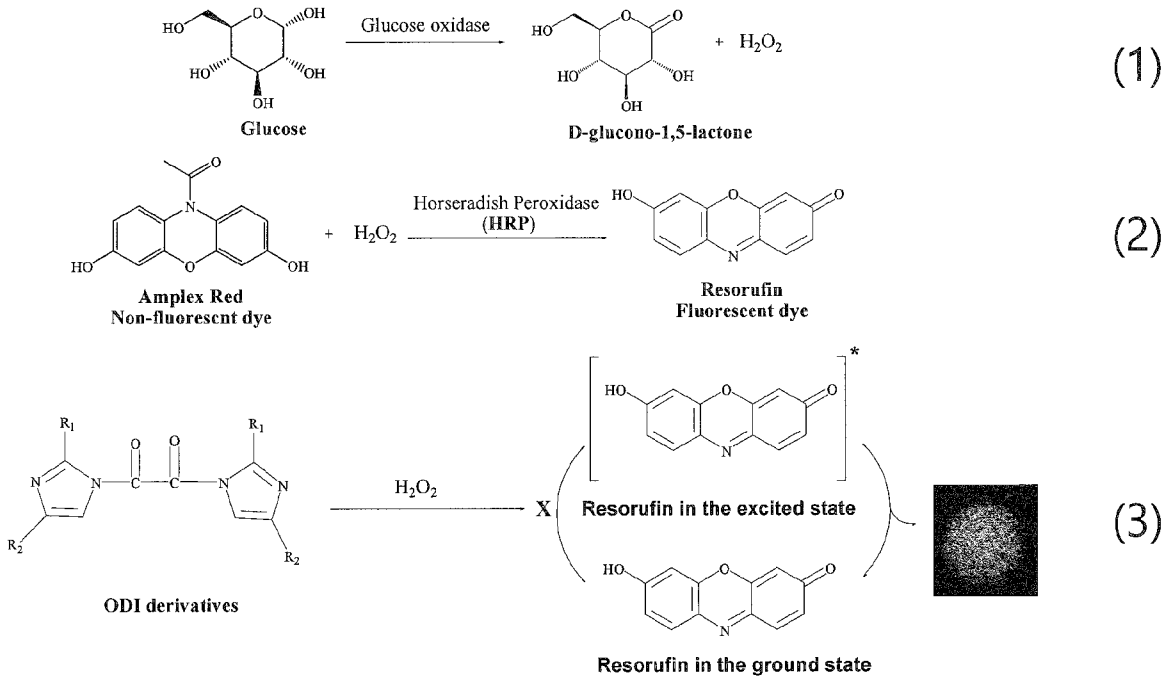


FIG. 2

FIG. 3

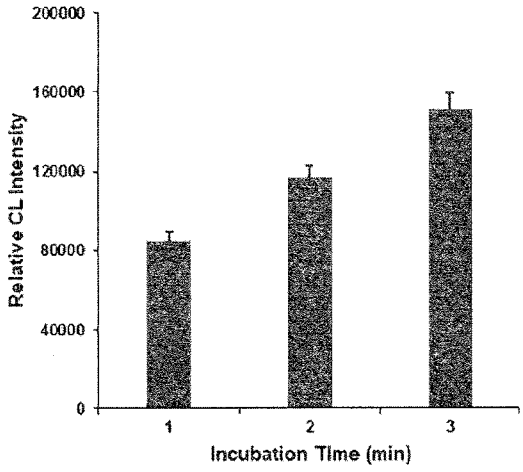


FIG. 4

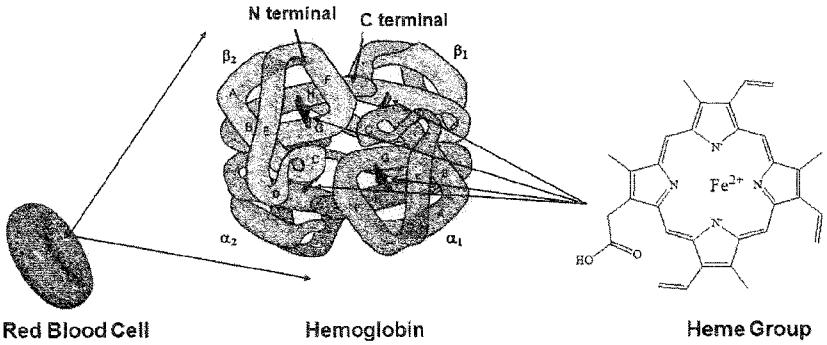

N terminal
C terminal
β2
β1
α2
α1
Fe2+
HO
Red Blood Cell
Hemoglobin
Heme Group

FIG. 5

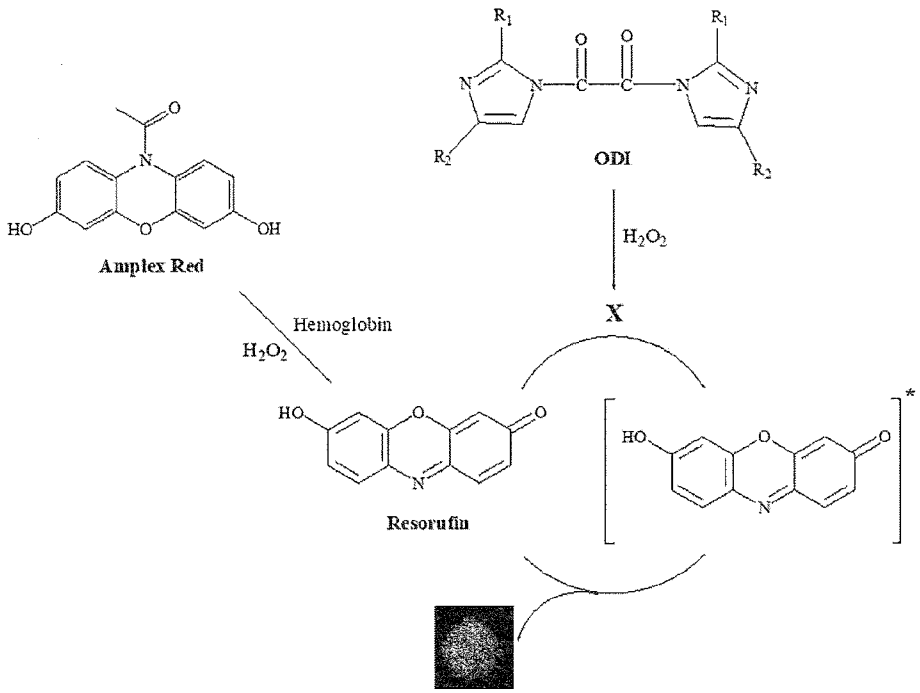

R1
R2
ODI
H2O2
X
HO
OH
Amplex Red
Hemoglobin
H2O2
Resorufin
*

FIG. 6

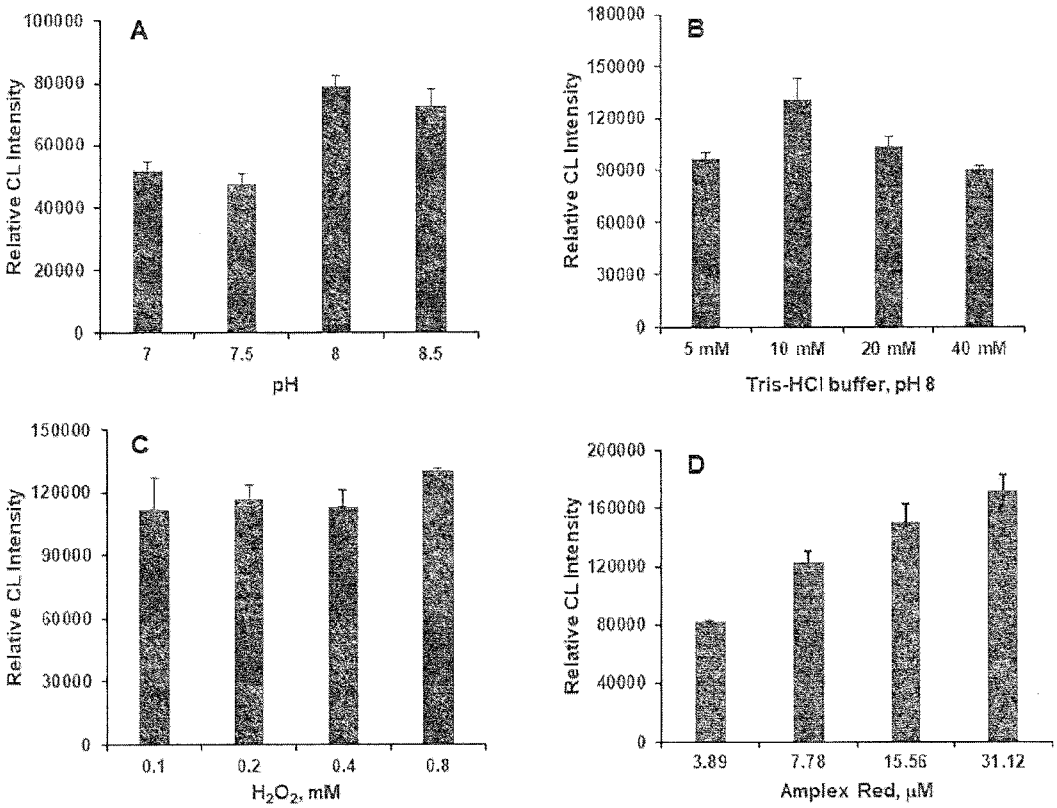

A
Relative CL Intensity
100000
80000
60000
40000
20000
0
7
7.5
8
8.5
pH
B
Relative CL Intensity
180000
150000
120000
90000
60000
30000
0
5 mM
10 mM
20 mM
40 mM
Tris-HCl buffer, pH 8
C
Relative CL Intensity
150000
120000
90000
60000
30000
0
0.1
0.2
0.4
0.8
H2O2, mM
D
Relative CL Intensity
200000
160000
120000
80000
40000
0
3.89
7.78
15.56
31.12
Amplex Red, µM

FIG. 7

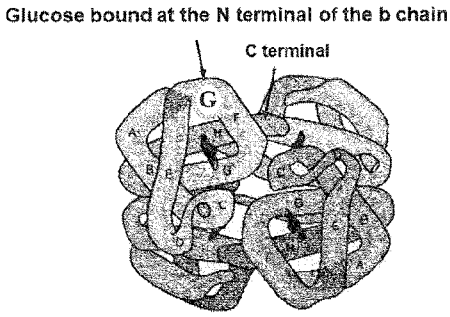

Glucose bound at the N terminal of the b chain
C terminal
G

FIG. 8

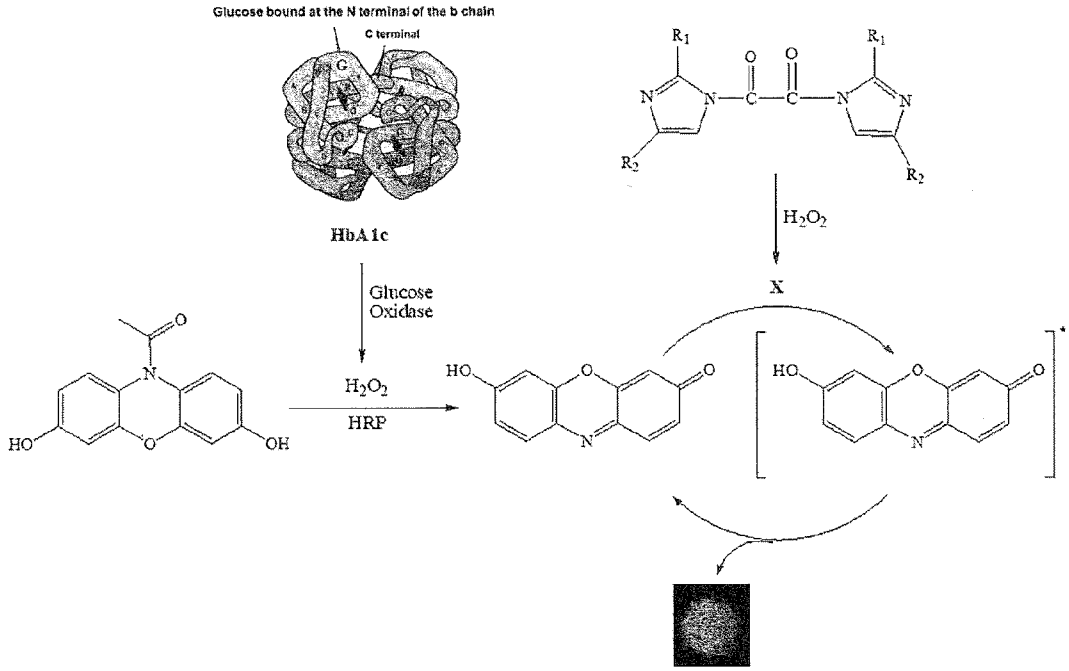

Glucose bound at the N terminal of the b chain
C terminal
HbA1c
Glucose Oxidase
H_2O_2
HRP
H_2O_2
X

FIG. 9

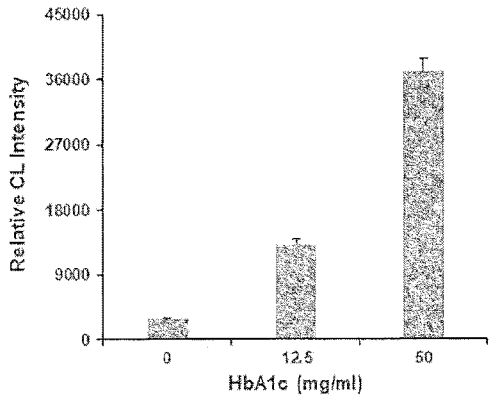

45000
36000
27000
18000
9000
0
Relative CL Intensity
0
12.5
50
HbA1c (mg/ml)

FIG. 10

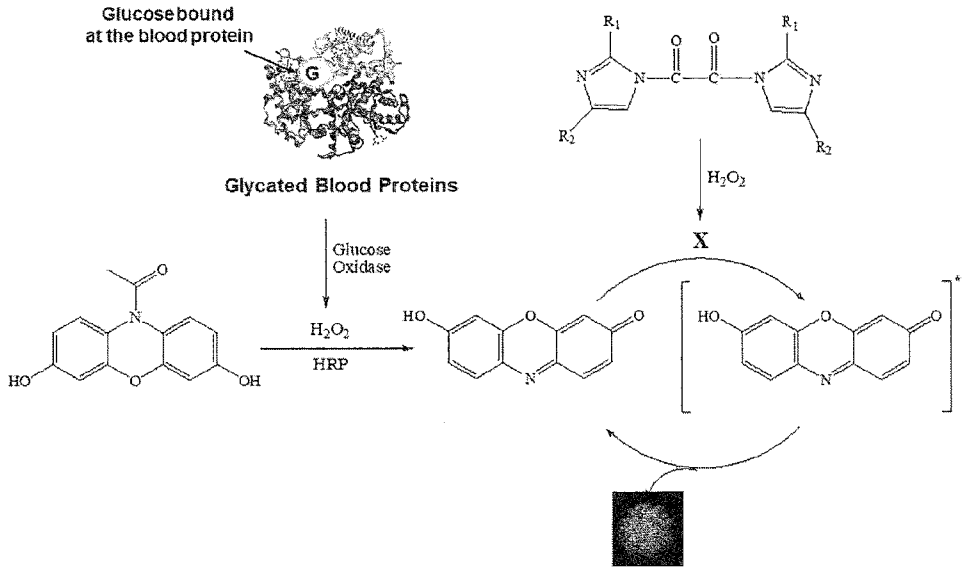


FIG. 11

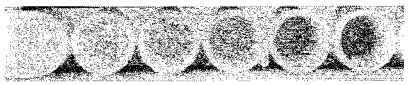
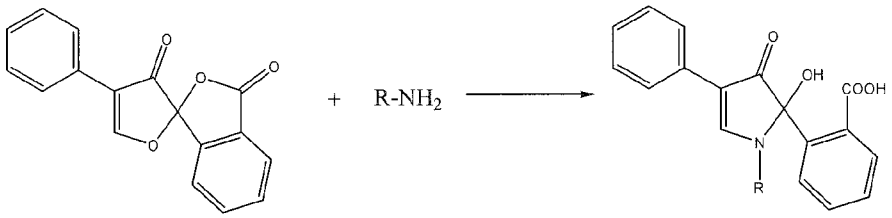


FIG. 12



Fluorescamine
Non-fluorescent dye

Fluorescent dye

FIG. 13

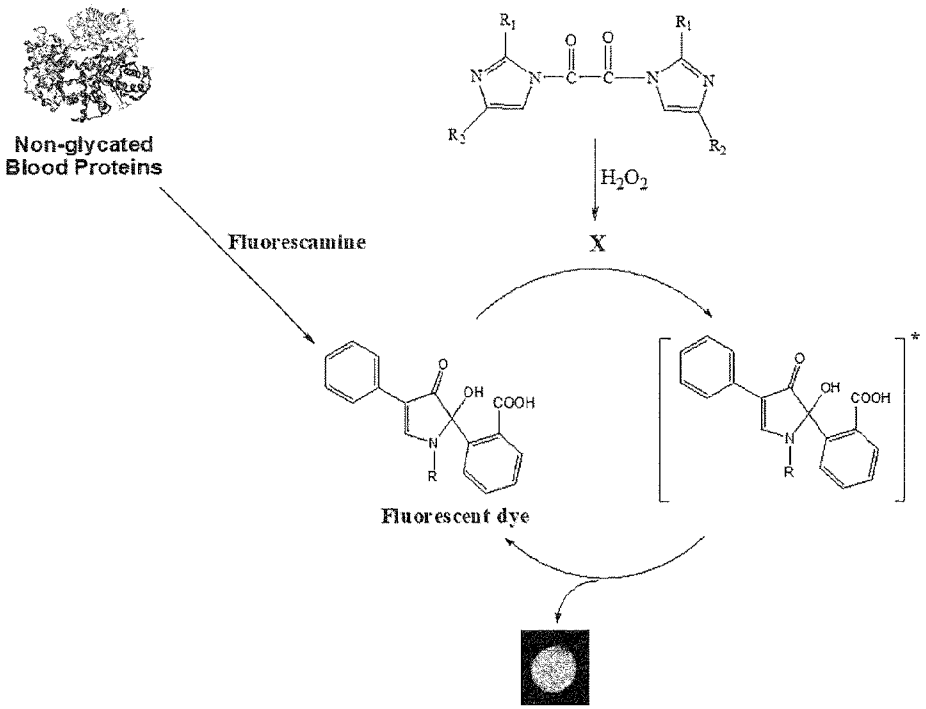

Non-glycated
Blood Proteins
Fluorescamine
H_2O_2
X
Fluorescent dye
OH
COOH
R
*

FIG. 14

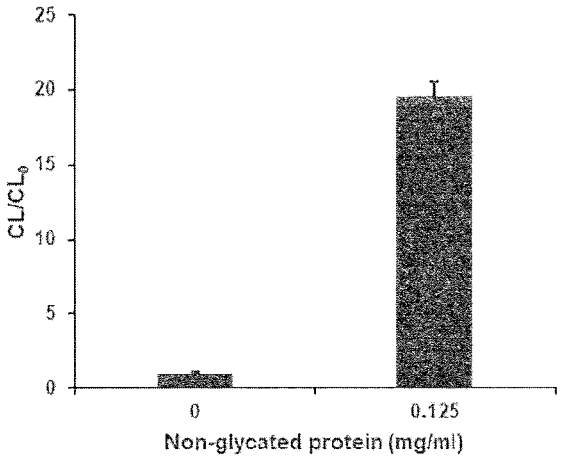

25
20
15
10
5
0
CL/CL_0
0
0.125
Non-glycated protein (mg/ml)

FIG. 15

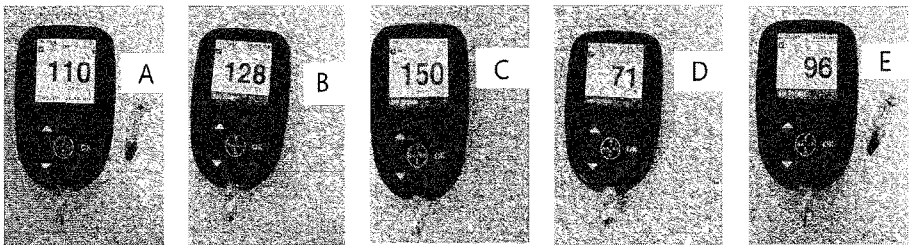

110
A
128
B
150
C
71
D
96
E

FIG. 16

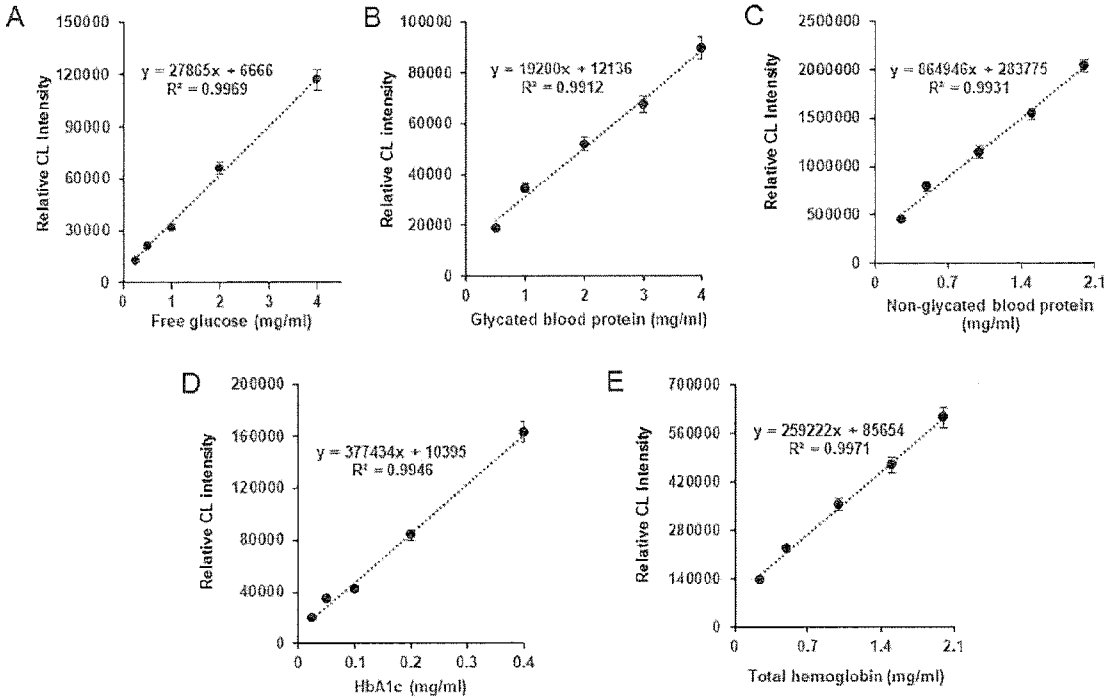

A
Relative CL Intensity
y = 27865x + 6666
R² = 0.9969
Free glucose (mg/ml)
B
Relative CL intensity
y = 19200x + 12136
R² = 0.9912
Glycated blood protein (mg/ml)
C
Relative CL Intensity
y = 864946x + 283775
R² = 0.9931
Non-glycated blood protein (mg/ml)
D
Relative CL Intensity
y = 377434x + 10395
R² = 0.9946
HbA1c (mg/ml)
E
Relative CL Intensity
y = 259222x + 85654
R² = 0.9971
Total hemoglobin (mg/ml)

FIG. 17.

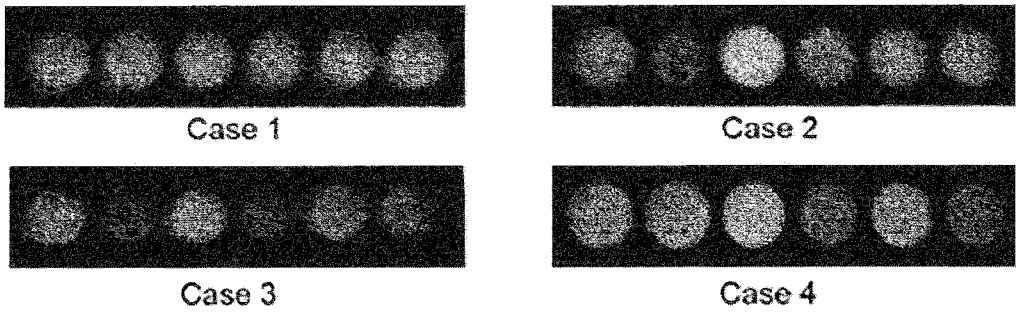
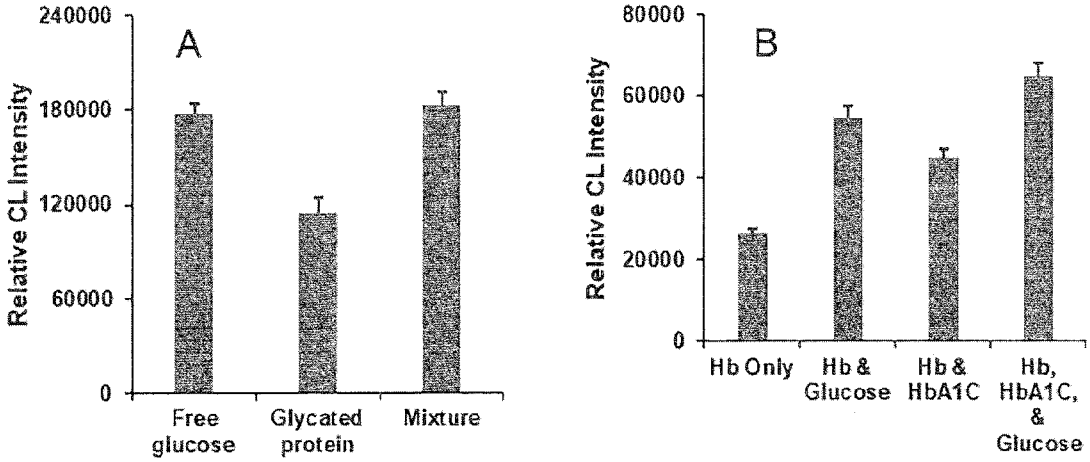

Case 1
Case 2
Case 3
Case 4

FIG. 18


A
Relative CL Intensity
240000
180000
120000
60000
0
Free glucose
Glycated protein
Mixture
B
Relative CL Intensity
80000
60000
40000
20000
0
Hb Only
Hb & Glucose
Hb & HbA1C
Hb, HbA1C, & Glucose