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A novel immobilization multienzyme glucose fluorescence capillary biosensor

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ABSTRACT

Based on the immobilization enzyme technology and the fluorescence capillary analysis method, the authors have developed a highly sensitive fluorescence reaction system and a novel immobilization multienzyme glucose fluorescence capillary biosensor for determining glucose contents. Reaction principle of the system is that under the catalysis of glucose oxidase (GOD) and horseradish peroxidase (HRP) immobilized on inner surface of a medical capillary, β-D-glucose reacts with dissolved oxygen to form gluconic acid-δ-lactone and hydrogen peroxide, and then the latter reacts with L-tyrosine to produce a tyrosine dimer, which has maximal excitation and emission wavelengths at 320 nm and 410 nm, respectively. Fluorescence of the dimer is proportional to the concentration of the β-D-glucose. Optimization conditions suitable for the reaction system and the biosensor were as follows. Concentration of the L-tyrosine used as fluorescence reagent was 0.15 mol L^{-1}, the active concentrations of the GOD and the HRP for the immobilization were 15 kU L^{-1} and 8 kU L^{-1}, respectively. Consumptions of the sample and reagents in one determination were 5.0 μL and 15 μL, respectively. Quantitative range of the biosensor for the glucose was in the range 1–10 μmol L^{-1}, its relative standard deviation was less than 4.9%, and its detection limit was 0.62 μmol L^{-1}. The biosensor's recovery was in the range 96–105%. Results of some serum determined with the biosensor and with a commercial glucose-kit were well coincident to each other. Accordingly, the biosensor can be applied to the determination of serum glucose contents in the diagnosis of diabetes.

1. Introduction

At present, incidence of diabetes mellitus which badly affects human being health is increasing. Under normal physiological case, the concentration of fasting plasma glucose is in the range 6.1–6.9 mmol L^{-1} and that of casual plasma glucose is less than 11.1 mmol L^{-1} (Alberti and Zimmet, 1998). So the level of the glucose content can indicate the probability of whether a human being is suffering from diabetes mellitus, hepatocirrhosis or psychopathy, and can give some important information for diagnosing diabetes mellitus etc. (WHO, 2006). Consequently, quantitation of the glucose content is paramount. Numerous methods for glucose analysis, such as spectrophotometry (Bateman and Evans, 1995; Edmiston and Williams, 2000; Vasilarou and Georgiou, 2000), amperometry (Cass et al., 1984; Gil et al., 1999), high performance liquid chromatography (Yuh et al., 1998), polarimetry (Luo et al., 2003), and capillary eletrophoresis (Frerichs and Colon, 1998) etc. have been reported. However, most of these methods are burdensome in operational procedure or expensive in cost. Consequently, it is necessary to develop a simple, sensitive, accurate, micro-volume and low-cost approach for glucose analysis which is appropriate for rapid field tests and is also effective as an alternative to the existing methods.

In early methods for the determination of glucose contents, most of them were based on the spectrophotometries containing free-enzymes. In 1957, for the first-time liquid glucose oxidase (GOD) was used for determination of the glucose (Free et al., 1957). In this method, under catalysis of GOD, glucose was oxidized to gluconic acid and H_2O_2, and then under catalysis of horseradish peroxidase (HRP), H_2O_2 reacted with o-tolidine reagent to form a blue dye, whose absorbance value was proportional to the concentration of glucose. Its detecting range was 5.6–220 mmol L^{-1}. Whereafter, phenol (Huang et al., 2001), N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (Han et al., 2003), α-naphthol-β-potassium sulfonate (Jiang et al., 2003) and antipyrine (Basak, 2007) as chromogenic reagents replacing the o-tolidine were also used to the above-mentioned reaction system, the detection limit of glucose reached 1.0 mmol L^{-1}. Because the sensitivity of these methods was lower and was only about thousandth of that of fluorophotometry, their application has been limited to micro-volume analysis of the glucose. Besides, as a lot of enzymes were consumed in using these methods, their assay costs were also higher.

Fluorophotometry has been widely used for the determination of glucose, owing to its higher sensitivity. Based on fluorescence

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intensity increasing principle, by using fluorescent matter formed in a reaction system of glucose/GOD/H_2O_2/hemoglobin/p-cresol, Wang et al. have quantified indirectly the glucose concentration at maximal excitation (320 nm) and emission wavelengths (419 nm) (Wang and Lu, 2002). Replacing p-cresol with pyronine B, Chen et al. (2003) have also attempted to develop another determining method for glucose.

Besides, on the basis of a principle that dissolved oxygen (DO) quenches the fluorescence of pyrene sulfonyl chloride ($\lambda_{ex/em}$: 347 nm/395 nm), Tatsu and Yamamura (2002) have also studied fluorescence measurement of glucose by pyrene-modified glucose oxidase. Hussain et al. (2005) have investigated measurements of the intrinsic fluorescence decrease of yeast hexokinase as an assay for glucose, and studied immobilization of the enzyme in a silica sol–gel matrix as a potential in vivo glucose sensor for use in patients with diabetes. Recently, Yang et al. (2006) have described an optical glucose sensor, which was based on the DO quenching the fluorescence produced by tris-(4,7-diphenyl-1,10-phenanthroline) rsuthenium (II) ditetrakis (4-chlorophenyl) borate. But its shortcomings were that the structure of this sensor was complicated, and bamboo's inner membrane used as the enzyme immobilization carrier was also easily ruptured.

Fluorescence capillary analysis (FCA) is a new method (Li and Gao, 2005). It is based on a general fluorophotometry and a medical capillary (ID: 0.50–0.90 mm, length: 3–5 cm) as well as a special capillary holder (Li and Gao, 2005). In FCA, the capillary (≤18 μL) replaces routine fluorescence cell (1–4 mL) and can save about 99% enzymes or reagents in comparison with the general fluorophotometry (Li and Gao, 2005). With the help of the FCA, we have successfully developed some special fluorescence biosensors for determination of alcohol in wines (Li and Gao, 2007a), lactic acid in blood (Li et al., 2008; Li et al., 2009), pyruvic acid (Zhao et al., 2008), sulfated bile acid in urine (Li et al., 2008a), and an immobilized probe biosensor marked by Cy-5 reagent for DNA (Gao et al., 2006).

In 2008, using cheap, non-toxic fluorescein as a fluorescent reaction substrate, we had proposed a liquid enzyme fluorescence capillary quenching method for determination of glucose in serum, based on the reaction system of glucose/GOD/H_2O_2/fluorescein/horseradish peroxidase (Du et al., 2008). However, later we have found that the reaction system cannot be used to make an immobilization enzyme glucose biosensor. The reason was that the fluorescein directly reacts with reagents used for immobilization (γ-aminapropyl triethoxy silane and glutaraldehyde) and led to its fluorescence pre-quenching.

Consequently, in this research we have screened other fluorescent reaction systems, such as the glucose/GOD/H_2O_2/o-phenylenediamine/HRP, glucose/GOD/H_2O_2/thiamine/HRP, and glucose/GOD/H_2O_2/L-tyrosine/HRP systems, etc. The study found that the L-tyrosine's fluorescence intensity in the system is stable and it does not react with reagents for immobilization. Therefore, we selected a new reaction system containing L-tyrosine as the fluorescent reaction substrate to make an immobilization enzyme glucose fluorescence capillary biosensor (IE-GFCBS). The merits of the IE-GFCBS were that it can overcome the deficiencies as mentioned above, simplify operating procedures, save lots of enzyme reagents, decrease waste, and achieve its reuse.

2. Experimental

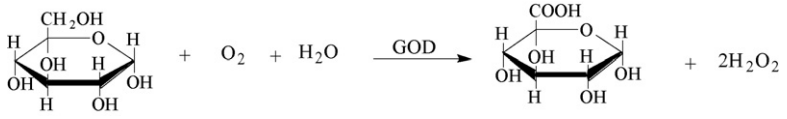
2.1. Chemicals and enzymes as well as apparatus

All chemicals used were analytical reagent grade without further purification. GOD (EC 1.1.3.4, 151 U mg^{-1}) and HRP (EC 1.11.1.7, 250 U mg^{-1}) were purchased from Sigma (St. Louis, MO, USA); glucose-kit was from Shanghai Institute of Biological Products (Shanghai, China); L-tyrosine ($C_9H_{11}NO_3$) was purchased from Shanghai Bio Life Science & Technology Co. Ltd. (Shanghai, China). β-D-glucose was purchased from Kelai organics Co. Ltd. (Chengdu, China). Ethanol, sodium hydroxide, γ-aminapropyl triethoxy silane (JH-A110 type silane coupling agent, 98%, v/v), glutaraldehyde($C_5H_8O_2$), tris were purchased from Baoxin Bioengineering Co. Ltd. (Chengdu, China). Disodium hydrogen phosphate and monosodium dihydrogen phosphate were bought from Chengdu chemical reagent Co. Ltd. (Chengdu, China). Commercial glucose-kits were bought from Baoding Great Wall Clinical Reagents Co. Ltd. (Baoding, China). Collected blood samples were fresh from volunteers, after overnight fasting.

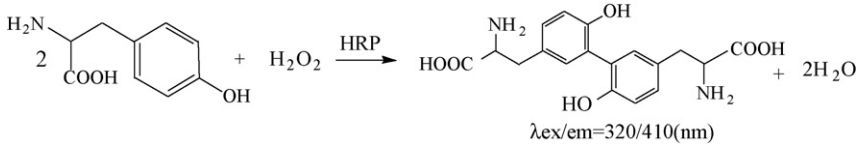
The apparatuses used in the research include a RF-5301 type spectrofluorimeter made by Shimadzu Co. (Kyoto, Japan), a pHS-3B type acidity meter of Shanghai Leici Equipment Company (Shanghai, China), an AUW120D type electric balance (measuring precision: 0.01 mg) made by Shimadzu Co. (Kyoto, Japan), a thermostatic drying chamber-202 from Rongfeng Experiment Co. Ltd. (Shanghai, China) and a 721-A type spectrophotometer from Shanghai third analyzer Co. (Shanghai, China). Besides, we made a homemade capillary holder (Li and Gao, 2007b) critically matched with the dark chamber of the above-mentioned spectrofluorimeter. The medical capillaries used (ID: 0.9 mm; OD: 1.1 mm) were purchased from Huaxi Instrument Factory of Sichuan University (Chengdu, China).

2.2. Analytical principle

Reaction principle of the IE-GLCBS for determining glucose is shown in the following Eqs. (1) and (2). Under catalysis of the GOD and the HRP immobilized on inner surface of the medical capillary, the glucose reacts with dissolved oxygen in water to form gluconic acid-δ-lactone and hydrogen peroxide, and then the latter reacts with L-tyrosine to create tyrosine dimmer with stronger fluorescence.



(1)



(2)

The fluorescence intensity is proportional to concentration of the glucose. Therefore its content can be indirectly determined by using the fluorescence.

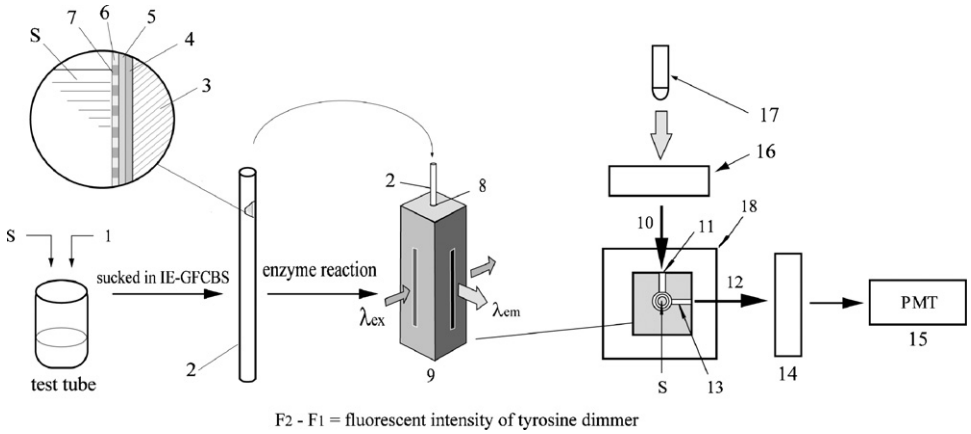


Fig. 1. Illustration of glucose determined with an IE-GFCBS. S, sample; (1) L-tyrosine solution; (2) IE-GFCBS; (3) inner surface of capillary; (4) γ-aminapropyl triethoxy silane; (5) glutaraldehyde; (6) GOD; (7) HRP; (8) inserting hole; (9) orientation holder; (10) excitation beam; (11) incidence window; (12) fluorescence; (13) emission window; (14) homochromy filter; (15) fluorescence detector; (16) fluorescent intensity controller; (17) light resource; (18) dark chamber; F_1, blank value; F_2, measure value.

2.3. Preparations of reagent solutions

Ultra-purified water (conductivity: 0.065 μS cm^{-1}) was used for preparation of following solutions.

2.3.1. Phosphate buffer solutions (0.10 mol L^{-1})

These solutions were prepared by weighing 35.81 g of Na_2HPO_4 in 1000-mL calibrated flask and 3.40 g of KH_2PO_4 in 250-mL calibrated flask, dissolving and diluting to the mark with water. Preparations of buffer solutions having different acidities were completed by mixing two phosphate solutions according to different proportions, respectively.

2.3.2. L-Tyrosine solution (5.0 mmol L^{-1})

45.3 mg of L-tyrosine was dissolved by using 5 mL of 0.5 mol L^{-1} NaOH and diluted to 50 mL by using phosphate buffer solution (pH 8.5).

2.3.3. Mixture solutions of GOD (8.0 kU L^{-1}) and HRP (15.0 kU L^{-1})

2.67 mg of the GOD and 3.00 mg of the HRP were exactly weighed and dissolved in a 50-mL calibrated flask, and diluted to the mark with phosphate buffer solution (pH 8.5).

2.3.4. γ-Aminapropyl triethoxy silane (2.5%, v/v)

The solution was prepared by taking 250 μL of γ-aminapropyl triethoxy silane solution in a 10-mL calibrated flask and completing the volume to the mark with water.

2.3.5. Glutaraldehyde solution (2.5%, v/v)

The solution was prepared by taking 250 μL of glutaraldehyde solution (50%, w/v) in a 5-mL calibrated flask and completing the volume to the mark with water.

2.4. Immobilization of GOD and HRP on inner surface of the capillary

Firstly, the inner and the outer surfaces of the capillaries were washed by using an ethanol solution containing 2 mol L^{-1} of NaOH, and flushed by using ultra-purified water. The capillaries pretreated were put in a thermostat for drying at 40 °C (Li et al., 2008b).

Secondly, they were aminated in a 2.5% (v/v) JH-A110 solution for 6 h at 55 °C, to make it covalently combine with hydroxyl group on surface of the capillaries, and were flushed by n-hexane and water solutions in turn. The purpose of this operation is that amidogens of JH-A110 were made in combination with hydroxyls on surface of the capillaries.

And then these capillaries were marinated in a 2.5% (v/v) glutaraldehyde solution as a cross-linker for 2 h on the vacuum condition to combine amidogen immobilized on the capillaries with aldehyde group of glutaraldehyde. Redundant glutaraldehyde solution was washed by using 0.1 mol L^{-1} of phosphate buffer solution (pH 7.0). In this way, the processing procedure of aldehyde group on surface of the capillaries was completed.

Thirdly, the capillaries sucked into a mixing solution consisting of the GOD and HRP (pH 8.5), kept overnight (12 h) at 4 °C. This operation is for the purpose that amidos of the GOD and HRP were chemically combined with aldehyde groups on surface of the capillaries and two enzymes were immobilized on the capillaries.

Finally, the capillaries were marinated in 0.05 mol L^{-1} Tris–HCl buffer (pH 7.0) for about 2 h to block out otiose aldehydic group. In this way the IE-GFCBSs were made. When not in use, they were stored in 0.05 mol L^{-1} PBS (pH 7.0).

2.5. Determination procedures

Determination procedure is shown in Fig. 1. First, a glucose standard solution (C_{st}) (50 μL) and L-tyrosine (50 μL) as well as PBS (100 μL) are mixed beforehand in a 500-μL test tube to form a fluorescent reaction mixture solution (FRMS). And then the FRMS in the test tube is sucked immediately into an IE-GFCBS (18 μL), and it is inserted in the top hollow of a holder which is in the spectrofluorimeter's dark chamber, and is detected to obtain a fluorescence intensity value (F_1) used as the blank value of the biosensor. Second, after taking out the IE-GFCBS from the holder and enveloping its two ends with two rubber caps, it is placed in a thermostat to continuously react 10 min. And then it is detected again to obtain a measure value (F_2) and a ΔF_{st} value ($\Delta F_{st} = F_2 - F_1$). The used biosensor can be regenerated after it was flushed by using the phosphate buffer.

The determination procedure of actual serum sample is the same with that of the glucose standard solution. After the same manipulation to the serum sample, another ΔF_s value ($\Delta F_s = F_2' - F_1'$) can be obtained. Finally, in the linear range of the biosensor response, the glucose content (C_s) in the serum sample can be calculated according to this formula: $C_s = (\Delta F_s/\Delta F_{st})C_{st}$. If utilizing a calibration curve method, ΔF_s is substituted by a linear equation of the glucose standard curve ($\Delta F_s = kC_s + b$) and is ready for finding the glucose content (C_s).

The function of the IE-GFCBS in the determination procedure was not to be only an immobilization carrier of the enzymes, but also a container that the sample reacts with reagents.

3. Result and discussion

3.1. Tyrosine dimmer scanning

In the glucose/GOD/HRP/L-tyrosine system, L-tyrosine dimer formed is an important fluorescence substance for the determination of glucose. To observe its maximal excitation and emission wavelengths, the mixture solution containing glucose/GOD/HRP/L-tyrosine was scanned by using the fluorophotometer at room temperature. Obtained result was shown in Fig. 2. It can be seen that obtained maximal excitation and emission wavelengths were 320 nm and 410 nm, respectively. So, two wavelengths were used in next experiments.

3.2. Effect of GOD concentration for immobilization on the biosensor

For further improving storage stability of GOD and HRP and reducing the test cost in fluorescence capillary analysis, we have attempted that the two enzymes were simultaneously immobilized on inner surface of capillary to form a sort of capillary biosensor. Therefore, enzyme concentrations used for immobilization were optimized under next experiments.

In the reaction system of the glucose/GOD/HRP/L-tyrosine, because at the first step, oxidation of glucose needs catalysis of GOD according to Eq. (1), GOD was a key enzyme and had an important effect on the reaction rate and sensitivity of the reaction system. So the effect of active concentration of the GOD for immobilization on the fluorescence intensity was investigated by using six biosensors which immobilized different active concentrations of GOD (2.5, 4.0, 5.0, 8.0, 10 and 20 kU L^{-1}).

The FRMS used in the test consists of 1.0 mmol L^{-1} of L-tyrosine (200 μL) and 2 kU L^{-1} of HRP (100 μL) and 0.1 mol L^{-1} of PBS (pH 8.5) (600 μL). 2.0 and 6.0 μmol L^{-1} of glucose standard solutions (100 μL) were used as test sample. Reaction temperature was 35 °C and reaction time was 20 min. The same tests were repeatedly conducted thrice. The results obtained were shown in Fig. 3. When the active concentration of the GOD was 8.0 kU L^{-1}, the fluorescence intensity attained the maximum. Consequently, the concentration for the immobilization in the biosensor was selected 8.0 kU L^{-1}.

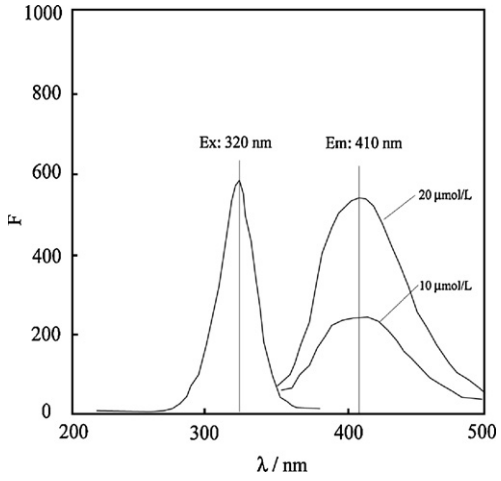


Fig. 2. Results obtained by scanning the tyrosine dimmer formed in the glucose/GOD/HRP/L-tyrosine reaction system.

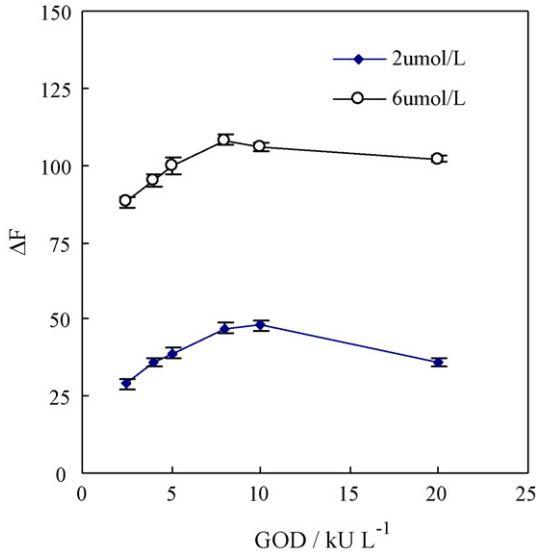


Fig. 3. Effect of the active concentration of GOD for immobilization on the capillary biosensor.

3.3. Effect of HRP concentration for immobilization on the biosensor

Because in Eq. (2), redox reaction between hydrogen peroxide generated and L-tyrosine needs catalysis of HRP, HRP was also another key catalyzer. So, the HRP's active concentrations to be used for the immobilization in the biosensor were examined.

In this experiment, the FRMS was prepared by using 1.0 mmol L^{-1} of L-tyrosine (200 μL) and 8.0 kU L^{-1} of GOD (100 μL) as well as 0.1 mol L^{-1} of PBS that its pH was 8.5 (600 μL). After amination and aldehydation of capillaries, the enzyme immobilization was conducted on them by using different active concentrations of HRP (2.5, 4.0, 5.0, 8.0, 10 and 20 kU L^{-1}). Other experimental conditions were as described in Section 3.2. Obtained result was shown in Fig. 4. It was obvious that the fluorescence intensity gradually increases with increasing the active concentration of HRP in the range 2.5–15 kU L^{-1}, and decreases after 15.0 kU L^{-1}. Therefore, HRP's active concentration for the immobilization on the capillaries was chosen 15.0 kU L^{-1}.

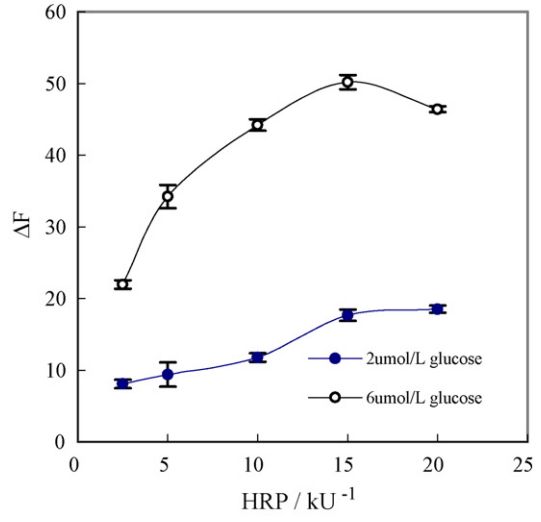


Fig. 4. Effect of the active concentration of HRP for immobilization on the capillary biosensor.

3.4. Effect of L-tyrosine concentration on the biosensor

In the reaction system, under catalysis of the GOD the glucose reacts with dissolved oxygen to form gluconic acid-δ-lactone and hydrogen peroxide, and then the latter reacts with L-tyrosine to create tyrosine dimmer with stronger fluorescence under catalysis of the HRP. The concentration of glucose can be indirectly quantified by detecting the fluorescence intensity of tyrosine dimmer. Consequently, the L-tyrosine concentration will affect on this enzymatic reaction system for glucose determination.

In the testing, 8.0 kU L^{-1} of GOD and 15 kU L^{-1} of HRP were used for the immobilization on inner surface of two capillaries. Other experimental conditions were as described in Section 3.3. Influence of the L-tyrosine concentration in the range 0.25–1.5 mmol L^{-1} on the response of the biosensor was investigated. FRMS was prepared by using 200 μL of L-tyrosine with different concentrations (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mmol L^{-1}) and 800 μL of PBS such that its pH is 8.5 and concentration is 0.1 mol L^{-1}.

Experimental results were shown that the fluorescence intensity of the biosensor increases with the increase of the L-tyrosine concentration, and reaches the maximum at 0.75 μmol L^{-1}, and becomes constant over 0.75 μmol L^{-1} (refer to Supplementary Material as shown in Fig. 5). To be on the safe side, 1.0 μmol L^{-1} of the L-tyrosine was selected to use.

3.5. Effect of acidity in reaction mixture solution on the capillary biosensor

Acidity in fluorescent reaction system containing enzymes is a very important factor because it can influence the catalytic rate of enzymes and quantum yield of fluorescence, so optimization was made to it. The experimental conditions were the same as described in Section 3.4, except that the L-tyrosine concentration used was 1.0 μmol L^{-1}. The FRMS was prepared by using 200 μL L-tyrosine and 800 μL PBS. Its acidity was changed by using PBS with different pH values (7.0, 7.5, 8.0, 8.0, 8.5, 9.0 and 9.5).

From results it can be seen that the fluorescence intensity reached the maximum when pH value in the FRMS was 8.5 (refer to Supplementary Material as shown in Fig. 6). So, this pH value was selected for next test.

3.6. Effect of temperature on the capillary biosensor

Analytical sensitivity of the capillary biosensor is interrelated with reaction temperature, so its effect on the fluorescence intensity was investigated. Experimental conditions were as described in Section 3.5, except that pH of the PBS was 8.5.

Ten immobilized biosensors with the same fluorescence blank values (F_1) were selected for the test. After these biosensors inhaled the same reaction mixture solution and reacted with it at 25, 30, 38, 45 and 50 °C for 10 min, their fluorescence intensities (F_2) were detected, respectively. The detected results indicated that the fluorescence intensities increase with the rising of the reaction temperature, and reach the maximum at 38 °C, and decrease when the temperature is over 38 °C (refer to Supplementary Material as shown in Fig. 7). This is due to the influence of the temperature on catalytic reaction of the GOD and HRP. On one hand the raising of the temperature quickens reactive rate, on the other hand, it can also cause denaturation of the enzymes. Accordingly, the reaction temperature of the biosensor was selected to be 38 °C.

3.7. Effect of reaction time on the biosensor

The selected reaction temperature was 38 °C, other experimental conditions were as described in Section 3.5. By detecting the fluorescence intensity of two biosensors at 0, 5, 10, 15, 20 and 25 min, respectively, the effect of reaction time was investigated on the glucose/GOD/HRP/L-tyrosine system. Experimental result showed that the fluorescence intensity increases with the increase of the reaction time. Over 10 min the fluorescence intensity becomes constant (refer to Supplementary Material as shown in Fig. 8). Therefore, the reaction time was selected to be 10 min.

3.8. Effect of other substances in serum on the biosensor

On the basis of optimization conditions mentioned above, substances influencing the determination of serum glucose were investigated. Owing to the GOD in the reaction system possesses specificity, sucrose and fructose in serums should do not interfere with the biosensor. But the experiment proved that the concentrations of ascorbic acid (AsA) and uric acid (UA) in serum interfered with the determination of the glucose biosensor in the ranges 28.4–79.5 μmol L^{-1} of AsA and 90–420 μmol L^{-1} of UA. This problem has been resolved by highly diluting (after 100-fold) the serum samples in a permissible range of analytical sensitivity. This can be proved according to the recovery data narrated in Section 4.3.

In addition, it was also considered that other substances existing in serums, such as Fe^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, etc. may bring the interference to determine glucose. Therefore, adding these substances into the glucose standard samples, their interferences were also examined by the biosensors. Added concentrations of Fe^{2+}, Cu^{2+}, Zn^{2+}, and Ca^{2+} into samples were in the range of 28.4–79.5 μmol L^{-1}, 14.1–17.3 μmol L^{-1}, 28.4–79.5 μmol L^{-1}, and 2.24–2.75 mmol L^{-1}, respectively. Experimental results showed that the added substances in the ranges had not interfered with the IE-GFCBS.

3.9. Stability examination of IE-GFCBS

To examine the storage stability, the fluorescence intensities produced by single biosensor were detected repetitiously, thrice per week for one month. According to these data obtained, relative stability of the capillary biosensor was calculated. The relative stability was defined as the ratio of fluorescence intensities ($F_1/F_{2,3,4,n}$) obtained at initial- and arbitrary-time detections, the ratio ($F_1/F_1 = 1$) of the fluorescence intensity obtained at first-time detection was presumed a hundred percent of the relative stability.

In testing, 6 μmol L^{-1} of the glucose standard containing the L-tyrosine and PBS was used as test samples, other experimental conditions were as described in Section 3.6. Obtained results showed that the relative activity of the biosensor was still greater than 90% after one month. Namely, the stability of the biosensor was satisfactory.

4. Determination of the sample

4.1. Calibration of glucose standard solution

The optimum conditions of the IE-GFCBS were as follows. The pH of the PBS was 8.5, reaction time was 10 min, and reaction temperature was 38 °C. Maximal excitation and emission wavelengths of tyrosine dimmer formed in the reaction system were 320 nm and 410 nm, respectively. The concentrations of HRP and GOD for the immobilization were 15 kU L^{-1} and 8 kU L^{-1}, respectively. Concentration of the L-tyrosine was 1.0 μmol L^{-1}. Under these conditions, six glucose standard solutions were determined by single IE-GFCBS, obtained determination range for glucose was in 1.0–10 μmol L^{-1}, its linear equation was $\Delta F_s = 10.61C_s - 1.55$ ($r = 0.9943$).

Table 1
Glucose concentrations in serum samples obtained by using the IE-GCBS ($n = 3$).

Sample	Dilution multiple	Mean value/ΔF_s	Concentration/μmol L^{-1}	Final concentration/mmol L^{-1} [*]
Serum-1	1000	67.96 ± 0.95	5.56 ± 0.077[a]	5.56 ± 0.077
Serum-2	1000	24.80 ± 1.10	4.36 ± 0.095[b]	4.36 ± 0.095
Serum-3	1000	22.16 ± 0.63	3.88 ± 0.040[c]	3.88 ± 0.040

[a] $\Delta F_s = 12.84C_s - 1.62$($r = 0.9985$).
[b] $\Delta F_s = 5.53C_s + 0.32$($r = 0.9997$).
[c] $\Delta F_s = 5.53C_s + 0.32$($r = 0.9997$).
[*] $C_s = [(\Delta F - b)/k] \times$ dilution multiple.

Table 2
Recovery determination of serum samples by the IE-GCBS.

Sample	Glucose concentration[a]/ mmol L^{-1}	Added concentration/ mmol L^{-1}	Calculated concentration/ mmol L^{-1}	Found concentration/ mmol L^{-1}	Recover std./ mmol L^{-1}	Recovery/%
Serum-1	2.78	2.00	4.78	4.82	2.04	102
		4.00	6.78	6.98	4.20	105
Serum-2	2.18	2.00	4.18	4.22	2.04	102
		4.00	6.18	6.23	4.05	101
Serum-3	1.94	2.00	3.94	3.87	1.93	96.5
		4.00	5.94	6.04	4.10	103

[a] The glucose concentrations of each serum samples were after diluting one multiple.

4.2. Determination of serum sample

4.2.1. Pretreatment of serum sample

Generally, in the serum, other interference substances also exist, like some intrinsic enzymes or the some substances possessing fluorescence. In order to remove these interferences and to simplify the operating procedure, examinations were conducted to two pretreatment methods by using some serum samples collected from the hospitals.

One of method is that 400 μL of trichloroacetic acid (4%) was added in 250 μL of the serum sample to deposit protein, and again 400 μL of the PBS was added in it, centrifuged at 4000 rpm for 10 min. The supernatant fluid was taken and prepared to pH 8.5 with NaOH solution, its final volume was diluted to 25 mL with the PBS. Serum samples pretreated by the means mentioned above were stored in a refrigeratory to be used as the test samples. Another method is that after the serum sample was diluted directly with the PBS by means of some diluting ratio, it was mixed with the L-tyrosine reagent and reacted.

Experimental results show that the two methods do not evident difference. Consequently, pretreatment way of directly diluting sample was adopted in following determination.

4.2.2. Determination of serum sample

For examining its practicability, the IE-GFCBS was employed to determine the glucose content (C_s) in some serum samples. Determination procedures were the same as described in Section 2.5. Obtained results were shown in Table 1.

4.3. Recoveries and comparison experiments

To evaluate accuracy of data obtained by using the IE-GFCBS, firstly, recovery determination was conducted by using the same serum samples. Different concentrations of glucose standard solutions were added respectively into these serum samples and their fluorescence intensities were detected. Obtained results were summarized in Table 2. It can be seen that recoveries were in the range 96–105% and satisfactory.

To further evaluate credibility of the biosensor, secondly, the concentration of glucose in the serum sample was again determined by using the commercial glucose-kit, which is based on a glucose/GOD/phenol/4-aminoantipyrine/POD reaction system and spectrophotometric principle.

According to the testing procedure of the glucose-kit method, a series of the glucose standard solution were detected and a relevant equation of the absorbance (ΔA) and the concentration (C_{GLU}) of the glucose standard solution was obtained: $\Delta A = 0.043\, C_{GLU} + 0.091$. And again using the same glucose-kit, the serum-1, -2 and -3 were detected, and the corresponding serum glucose values were obtained and they were 5.40 ± 0.10 mmol L^{-1}, 4.22 ± 0.14 mmol L^{-1} and 3.64 ± 0.08 mmol L^{-1}, respectively. On comparison with the data in Table 1, it can be seen that data obtained by using the IE-GCBS and the glucose-kit were consistent. Therefore, this experimental result once again proved that our glucose biosensor is reliable in determining the actual serum samples.

4.4. Reproducibility and detection limit

To verify precision of the IE-GFCBS, 6 μmol L^{-1} of glucose standard solution was used as the test samples to repeatedly conduct fluorescence detections for eleven times. The mean value of the fluorescence intensity (ΔF) and the standard deviation (σ) obtained was 58.72 ± 2.88. After computing, relative standard deviation of the biosensor obtained was 4.91%. It can be seen that its reproducibility was satisfactory. Besides, by the regression equation of glucose calibration curve ($\Delta F_s = 14.0C_s - 0.12$, $r = 0.9943$), detection limit (C_L) of the IE-GFCBS was obtained 0.62 μmol L^{-1} ($C_L = 3\sigma/k = 3 \times 2.88/14.0 = 0.62$ μmol L^{-1}).

5. Conclusions

A novel and highly sensitive IE-GFCBS was successfully developed for the determination of glucose in serum, based on a new glucose/GOD/H_2O_2/L-tyrosine/HRP reaction system. Obtained optimization conditions were as follows: concentrations of the GOD and the HRP for immobilization were 8.0 kU L^{-1}, and 15 kU L^{-1}, respectively; the concentrations of phosphate buffer solution and the L-tyrosine solution were 0.15 mol L^{-1} and 1.0 μmol L^{-1}, respectively; the reaction time and temperature were 10 min and 38 °C, respectively. The biosensor's determination range was 1–10 μmol L^{-1}, relative standard deviation was less than 4.91%, and detection limit was 0.62 μmol L^{-1} for glucose standards. The

recoveries obtained by adding the glucose standards in serum samples were in the range 96–105%. Serum glucose values obtained by using the biosensor and the commercial glucose-kit have a good consistency with each other. Accordingly, the biosensor could be used for the determination of serum glucose contents in the diagnosis of diabetes.

By using our biosensor and the method, the reaction solution volume consumed was only 20 μL per one determination, and was 1/400 of traditional fluorescence method. So, the test cost can decrease to a great extent. The operation procedure of the biosensor and the apparatus adopted were simple, so it can be easily popularized. Following this preliminary report, we further attempt to estimate clinical samples from diabetes patients with the biosensor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.10.035.

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