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Glucose concentration determination based on silica sol–gel encapsulated glucose oxidase optical biosensor arrays

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ABSTRACT

Optical biosensor arrays for rapidly determining the glucose concentrations in a large number of beverage and blood samples were developed by immobilizing glucose oxidase (GOD) on oxygen sensor layer. Glucose oxidase was first encapsulated in silica based gels through sol–gel approach and then immobilized on 96-well microarrays integrated with oxygen sensing film at the bottom. The oxygen sensing film was made of an organically modified silica film (ORMOSIL) doped with tris(4,7-diphenyl-1,10-phenanthroline) ruthenium dichloride ($Ru(dpp)_3Cl_2$). The oxidation reaction of glucose by glucose oxidase could be monitored through fluorescence intensity enhancement due to the oxygen consumption in the reaction. The luminescence changing rate evaluated by the dynamic transient method (DTM) was correlated with the glucose concentration with the wide linear range from 0.1 to 5.0 mM ($Y = 13.28X - 0.128$, $R = 0.9968$) and low detection limit (0.06 mM). The effects of pH and coexisting ions were systemically studied. The results showed that the optical biosensor arrays worked under a wide range of pH value, and normal interfering species such as Na^+, K^+, Cl^-, PO_4^{3-}, and ascorbic acid did not cause apparent interference on the measurement. The activity of glucose oxidase was mostly retained even after 2-month storage, indicating their long-term stability.

1. Introduction

In recent decades intense interest has been concentrated on the development of glucose biosensor due to their application in diagnosis of diabetes and food safety inspection [1–12]. Until now two main sensors for glucose measurement have been explored, enzymatic and nonenzymatic. For the enzyme-free sensors, which were based on electrocatalytic oxidation of glucose directly on the electrodes, improving the sensitivity, selectivity, using of reference electrode and overcoming the interference from the anion (Cl^-) and ascorbic acid, uric acid became the main challenges faced during the development [3,4,6–8,11]. Constructing optical enzymatic glucose biosensors may provide an effective approach to overcome these problems because of the enzymatic specificity and high efficiency, which was less disturbed by interference species [1,2,9,10,12–15]. However, most of the optical glucose biosensors developed so far could undertake only one detecting mission at a time, and they also needed recovering time for the next measurement, limiting the throughput of sample detection. Therefore, exploring an efficient system amenable to simultaneous assays is an urgent problem for glucose biosensors.

On the other hand, finding an appropriate matrix to immobilize the enzyme and keep its activity in the enzymatic sensor for long-term application is another key issue. Silica gel [9,15–20], polymer [7], biomaterials [10,12], and their composites have been extensively explored to encapsulate enzymes and microorganism. Although great progress has been made, it still faces some challenges, such as leakage of enzyme from the matrix, complicity of the procedures for preparation of matrix, and short-term inheritance [1,2].

In this study, a glucose biosensor array was fabricated in 96-well array by using sol–gel method for encapsulating glucose oxidase and combining it with oxygen sensing film at the bottom of wells. These glucose optical biosensor arrays can be used to massively parallel assays up to 96 samples (and possibly more) using a commercial fluorescence plate reader. Glucose oxidase was immobilized in TEOS derived sol–gel, which was removed most of ethanol produced in hydrolysis by vacuuming. The luminescence intensity changing resulted from oxygen quenching was correlated with the glucose oxidation catalyzed by glucose oxidase. The glucose solution with different concentration was detected by using the glucose biosensor array and the dynamic transient method (DTM) was used to evaluate the luminescence kinetic graph. The normal interfering

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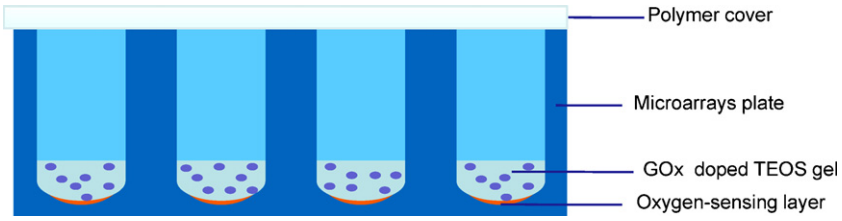

Fig. 1. The schematic illustration of the glucose oxidase encapsulated TEOS gel in microarrays integrated with oxygen sensing film.

factor such as high concentration sodium chloride, ascorbic acid was discussed. In addition, the long-term stability of glucose oxidase in the gel was evaluated by keeping the samples for 2 months.

2. Experimental

2.1. Apparatus

All the luminescence measurements were performed on a microplate reader Fluoroskan Ascent CF (Thermo Scientific) controlled by the software Ascent version 2.4 with the excitation and emission wavelength 400 and 620 nm, respectively. The top mode was chosen for measurements due to the transparency of the silica gel.

2.2. Chemicals

Glucose oxidase was purchased from Biozyme Laboratory Ltd., code GO3A (276 Unit/mg). Tetraethyl orthosilicate (TEOS) and triethoxy (octal) silane (octyl-triEOS) were purchased from Sigma–Aldrich. Tris(4,7-diphenyl-1,10-phenanthroline) ruthenium dichloride ($Ru(dpp)_3Cl_2$) was purchased from Alfa Aesar. Ethanol, methanol and concentrated hydrochloric acid were obtained from Wako. Potassium Dihydrogenphosphate were purchased from Nacalai tesque. 96 Microwell plates with round bottom were purchased from NUNC. Milli-Q water with the resistivity higher than 18 MΩ cm was used to prepare the aqueous solution. All chemicals and solvents were analytical-reagent grade and used without further purification.

2.3. Preparation of oxygen sensing film on microplate

The fluorophore doped sol was prepared as described in the following reference with small modification [21]. Briefly, 0.29 mL TEOS was mixed with 0.612 mL octyl-triEOS, 0.625 mL ethanol and 0.2 mL of 0.1 M HCl with magnetically stirring 1 h under ambient condition. And then 1.725 mL ethanol was added into the solution for diluting the sol to improve the quality of the oxygen sensing film. The solution was kept stirring for 1 h. To prepare $Ru(dpp)_3Cl_2$ doped sol, 100 μL of 2 mM $Ru(dpp)_3Cl_2$ in ethanol was mixed with 300 μL of the above mentioned sol solution. These solutions were capped and stirred for 30 min and 10 μL the fluorophore doped sol was pipetted into each well of microplate. The thickness of the oxygen sensing layer was less than 10 μm based on the amount of the sol/dye mixture deposited on the bottom. The microplate was stored in the dark under ambient condition for gelling and aging for 6 days.

2.4. The encapsulation of glucose oxidase in the TEOS gel

TEOS sol was prepared by mixing 0.5 mL TEOS, 0.25 mL deionized water and 12.5 μL of 0.1 M HCl and stirring for 3 h to form the homogeneous sol. The silica sol was vacuumed for 10 min to remove most of ethanol produced in the hydrolysis reaction. Then the sol was diluted four times by pure water. 500 μL Diluted sol was mixed with 500 μL of 20 mg/mL glucose oxidase solution dissolved in 0.1 M KPB. 30 μL of the above mixed sol was quickly pipetted onto the surface of the oxygen sensing layer in each well of the microplate. The microplate was kept in the refrigerator at 4 °C. A schematic illustration of glucose oxidase encapsulated agarose gel in the oxygen sensing microarrays is shown in Fig. 1.

2.5. Measurement of glucose solution with different concentrations using GOD microarrays biosensor

For the measurement of glucose solution, glucose solutions with different concentrations (0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 3.0, 4.0, 5.0, 8.0, 20.0 mM) were prepared by using 0.1 M KPB buffer solution. A 250 μL portion of the standard solution was added to each well of the microplates. A transparent microplate sheet (PET, Kajixx Ltd.) was used to seal the plate and prevent the oxygen in the air from diffusing into the microwells. After the addition of the substrate solution into the microarrays, the microplate was quickly placed on the platform of the microplate reader for the fluorescence measurement. Fluorescence intensity was recorded every 1 min for 25 min.

3. Results and discussion

3.1. GOD biosensor arrays response towards the glucose solution

Fig. 2A shows a typical kinetic response of glucose biosensor array for 2.0 mM glucose solution. The error bar represents the standard deviation ($n = 5$). A distinct fluorescence intensity increase was observed after the addition of glucose solution into the biosensor arrays. It indicated that TEOS derived gel retained the activity of glucose oxidase well. Oxygen consumption in the reaction reduced quenching of the luminescence from $Ru(dpp)_3Cl_2$ and resulted in an increase of fluorescence intensity. The fluorescence intensity arrived at the saturated state after exhausting oxygen or glucose. Interestingly, the kinetic courses of the fluorescence intensity change empirically fit well to the following sigmoid equation (fitting coefficient: 0.99).

$$I_t = \frac{I_1 + (I_0 - I_1)}{1 + e^{(t - t_{\max})/dt}}$$

I_t is the fluorescence intensity at the time t. I_0 and I_1 represents the fluorescence intensities at the beginning and after reaching the saturation, respectively. t_{max} represents the time point where the maximum rate of fluorescence intensity increase was observed (the time point of inflection). dt is the time range for the most significant fluorescence changes, which is used solely for the fitting purpose. We used this equation to fit the data points and derived the rate of fluorescence changes. The rate of fluorescence changes should reflect the reaction rate, and has been widely used to estimate the value of biosensor, significantly reducing the measurement time [9,12,14,17,19,20,22]. Therefore, we also employed the luminescence changing rate for the evaluation of kinetic behaviors (Fig. 2B) (the analytical technique is generally called as DTM) [9,12,14,22]. Although DTM is an empirical method, it can describe the complex kinetic responses of optical biosensors including substrate penetra-

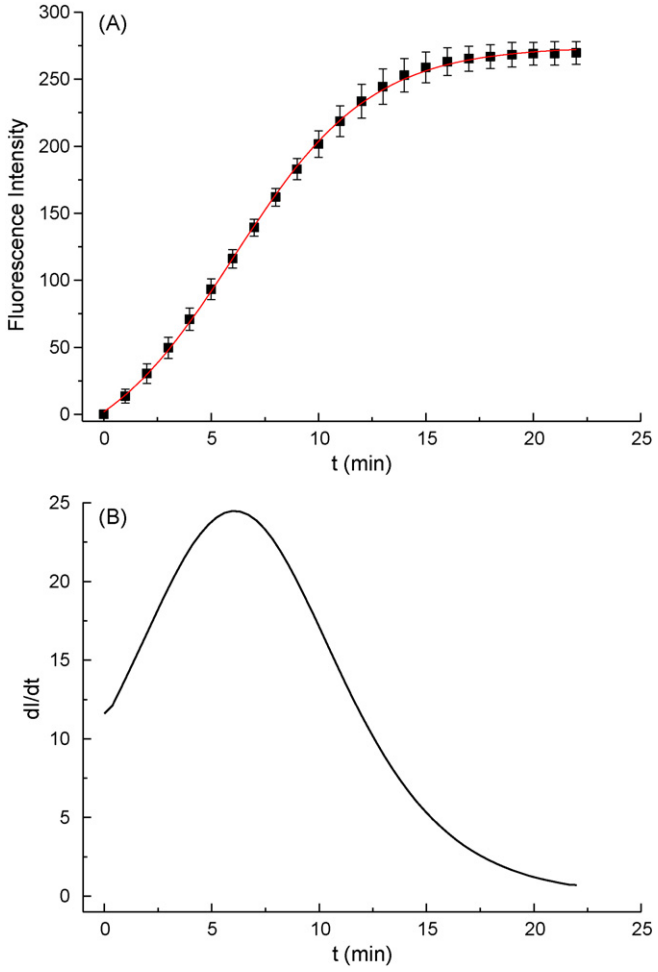

Fig. 2. The typical fluorescence responses of glucose biosensor arrays towards 2.0 mM glucose solution: (A) kinetic graph of fluorescence intensity and (B) the changing rate of fluorescence derived from (A).

tion into the gel, oxygen consumption by the enzymatic reaction, and oxygen diffusion/depletion in the gel. The maximal fluorescence changing rate appeared after 6 min observation, and we used it for evaluating the glucose concentrations.

3.2. Responses of GOD biosensor arrays towards different glucose concentration

Glucose biosensor arrays were used to evaluate the glucose solution with different concentrations. Fig. 3A shows the fluorescence changes for different glucose concentrations (0.1–20 mM). The fluorescence change with time was larger for higher concentrations of glucose. Fig. 3B summarized the maximum fluorescence changing rate as a function of the glucose concentration. Experiment results showed that glucose concentration from 0.1 to 5 mM has a linear relationship ($Y = 13.28X - 0.128$) with the maximum fluorescence changing rate with high coefficient to 0.9968. The limit of detection was calculated to be 0.06 mM from 3 times signal to noise. It is deviated the linear relationship when the concentration is above 5 mM as shown in the inset of Fig. 3B, which suggests the saturation of glucose oxidase catalysis under high concentrations of glucose. The evaluation of the fluorescence responses to different concentrations of glucose was done using parallel measurements of up to 25 wells. This clearly demonstrated the potential of this approach to be used in massively parallel assays.

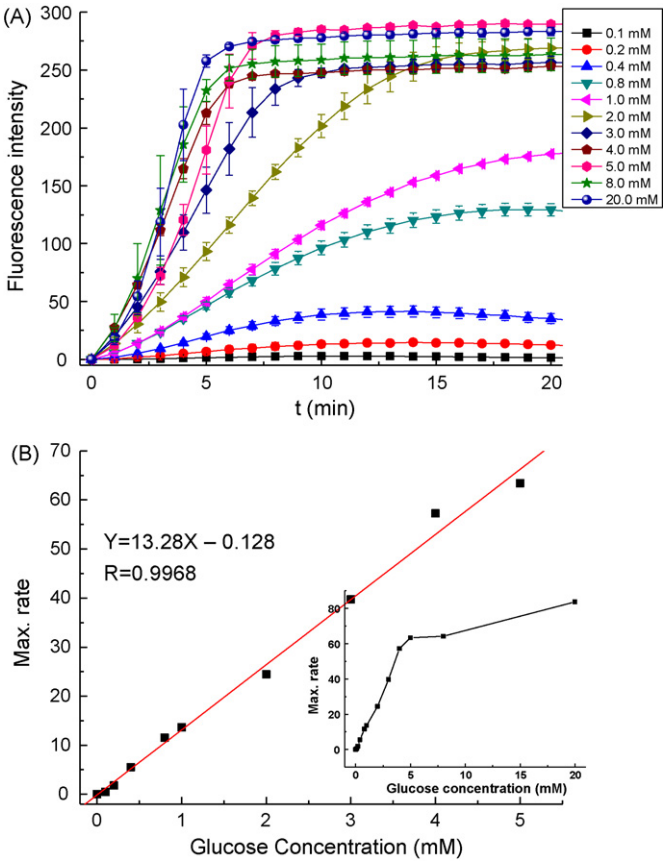

Fig. 3. (A) Fluorescence responses of glucose biosensor arrays towards different concentrations of glucose. (B) Correlation between the max luminescence rate and the concentration of glucose.

3.3. Effect of pH

It is well known that the activity of glucose oxidase in biosensor is quite sensitive to pH. Therefore, the effect of pH on the activity of glucose biosensor arrays was investigated over the range from pH 3 to 10. Fig. 4 shows the normalized response rate of the biosensor arrays against pH when the biosensor arrays were subjected

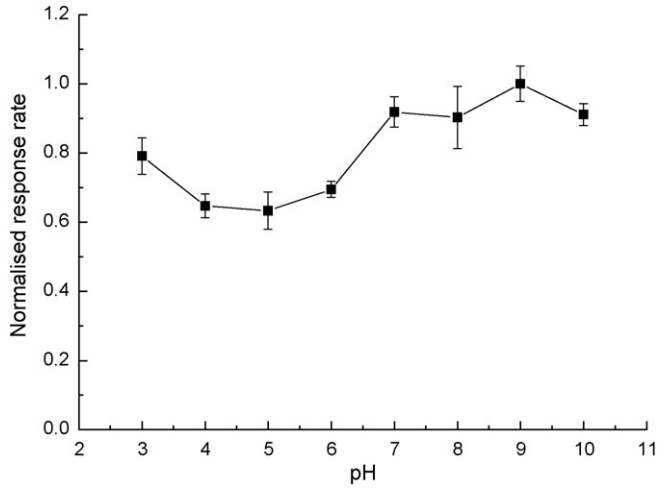

Fig. 4. Effect of pH on the glucose biosensor arrays by subjecting to 2 mM glucose in 0.1 M KPB solution. The relative response (%) was calculated by normalizing the signal with respect to the maximum signal. Each data was obtained from an average value of three replicate measurements.

Table 1
Determination of glucose contents in real samples and recovery test for glucose injection samples using the glucose biosensor arrays.

Sample	Concentration		Concentration of glucose added (mM)	Concentration of glucose found (mM)	Recovery (%)	R.S.D. (%)
	Proposed concentration (g/L)	Glucose biosensor arrays (g/L)				
5% Glucose injection[a]	50.4	49.95	–	–	–	4.2
			0.500	0.514	102	5.5
			2.000	2.120	106	7.2
20% Glucose injection[b]	198.0	202.5	–	–	–	6.0
			0.500	0.505	101	8.1
			2.000	2.200	110	9.4

R.S.D.: relative standard deviation.
[a] The sample was diluted 100 times for measurement.
[b] The sample was diluted 500 times for measurement.

to 2 mM glucose solution in 0.1 M KPB solution at various pH. The response rate can be remained above 90% for pH 7–10 and 60% for pH 3–6. It indicated that the changes in pH value cannot significantly affect the activities of biosensor arrays, especially in mild alkaline environment.

3.4. Interferences effect on the activity of GOD biosensor arrays

In the process to analyze the glucose in blood, ascorbic acid and chloride ion are the main interferences in the detection. Therefore, we investigated the effects of different ions (Na^+, K^+, Cl^-, PO_4^{3-}) and ascorbic acid under a high concentration of 0.1 M on the glucose biosensor arrays. No distinct interference was observed after the addition of interfering ions, suggesting that the glucose biosensor arrays possessed an excellent selectivity and specificity compared with the electrochemical nonenzymatic glucose sensor (see Supporting Information 1).

3.5. Repeatability and stability of GOD biosensor arrays

To evaluate the repeatability of glucose biosensor arrays, four continuous experiments were performed to evaluate 2 mM glucose solution by using the same five wells in the arrays. It was shown that no distinct changes happened to the biosensor arrays after 4 continuous detections, suggesting the excellent repeatability of the biosensor assays (see the Supporting Information 2). In Fig. 5, it compared the behavior of glucose biosensor arrays for 2 mM glucose solution after the storage for 0, 30 and 60 days at 4 °C. Slight decrease of the activity (4.6%) was found after 30 days keeping and only 10.4% signal decreased after 60 days keeping, which assured the long-term stability of the biosensor arrays.

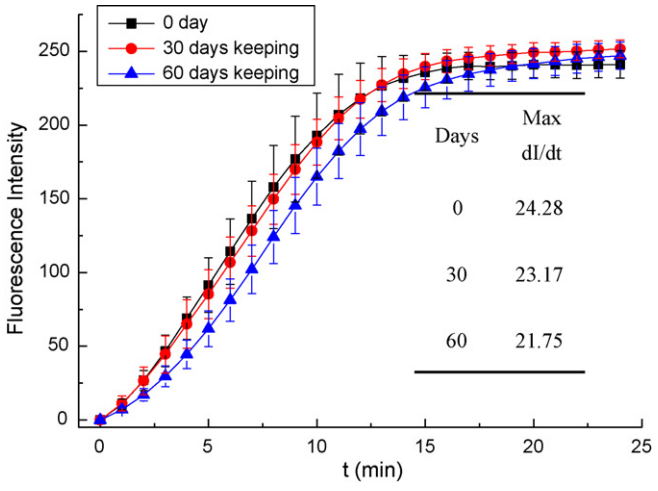

Fig. 5. Repeatability and stability test (30 days and 60 days keeping) on the glucose biosensor arrays.

3.6. Factual sample analysis

The high-throughput glucose biosensor arrays were applied to the analysis of glucose samples (commercially available glucose solution with known concentrations). The factual samples were diluted before measurement, 100 times dilution for 5% glucose injection and 500 times dilution for 20% glucose injection. The results obtained from the glucose biosensor arrays agreed with the suggested value on the samples (Table 1), indicating the results were accurate and credible. Recovery tests for glucose in injection samples were carried out by adding known amounts of glucose to the diluted injection samples. The results demonstrate that the recovery rate ranged from 102–110%. It confirmed the glucose biosensor arrays had an excellent performance for glucose detection in the factual samples.

4. Conclusion

Glucose biosensor arrays were developed by combining an oxygen sensing film with immobilized glucose oxidase in silica gel. The biosensor arrays showed different responses in accordance with the concentrations of glucose. The concentration of glucose in a wide range (from 0.1 to 5.0 mM) showed a linear correlation with the maximum fluorescence changing rate determined by the evaluation of DTM. As a significant advantage of this method compared with the electrochemical nonenzymatic method, no obvious interference was observed under a high concentration of chloride ion and ascorbic acid (0.1 M). The activity of glucose oxidase doped in silica gel was retained well for 1 month and no obvious changing was observed after 2-month storage, indicating an excellent stability of the biosensor arrays. In the present study, up to 25 samples could be simultaneously detected, but the number of samples assayed can be extended easily, vastly improving the throughput. The use of 96-well plates (or 384-well plates) enables efficient evaluation of glucose concentrations using commercial fluorescence plate readers. This should facilitate the glucose concentration determination in various settings such as beverage factories or clinical labs, where a large number of samples must be routinely handled. Furthermore, the present approach is applicable also to other enzyme immobilization with different sensing applications in a massively parallel fashion.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.08.039.

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