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Biosensors for determination of glucose with glucose oxidase immobilized on an eggshell membrane

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Abstract

A glucose biosensor using an enzyme-immobilized eggshell membrane and oxygen electrode for glucose determination has been fabricated. Glucose oxidase was covalently immobilized on an eggshell membrane with glutaraldehyde as a cross-linking agent. The glucose biosensor was fabricated by positioning the enzyme-immobilized eggshell membrane on the surface of a dissolved oxygen sensor. The detection scheme was based on the depletion of dissolved oxygen content upon exposure to glucose solution and the decrease in the oxygen level was monitored and related to the glucose concentration. The effect of glutaraldehyde concentration, pH, phosphate buffer concentration and temperature on the response of the glucose biosensor has been studied in detail. Common matrix interferents such as ethanol, D-fructose, citric acid, sodium benzoate, sucrose and L-ascorbic acid did not give significant interference. The resulting sensor exhibited a fast response (100 s), high sensitivity (8.3409 mg L^{-1} oxygen depletion/mmol L^{-1} glucose) and good storage stability (85.2% of its initial sensitivity after 4 months). The linear response is 1.0×10^{-5} to 1.3×10^{-3} mol L^{-1} glucose. The glucose content in real samples such as commercial glucose injection preparations and wines was determined, and the results were comparable to the values obtained from a commercial glucose assay kit based on a spectrophotometric method.

Keywords: Glucose biosensor; Eggshell membrane; Glucose oxidase

1. Introduction

Glucose is a major component of animal and plant carbohydrates. Quantitative determination of glucose is of paramount importance in biochemistry, clinical chemistry, and food analysis. Numerous methods such as spectrophotometry [1–5], amperometry [6,7], HPLC [8], polarimetry [9], and capillary eletrophoresis [10] for glucose analysis have been reported. However, most of the current adopted methods are lengthy or costly. In view of the increasing concern over quantitative determination of glucose there appears to be a need for new methods of glucose analysis appropriate for rapid field tests and also as an alternative to the existing methods. Recently, research in the development of chemosensors and biosensors has been very fast growing and many glucose biosensors have been presented.

A biosensor, a device incorporating a biological molecular recognition component connected to a transducer, can output an electronic signal proportional to the concentration of the analyte being sensed [11]. The high selectivity in biosensors provided by the biological recognition systems including antibodies, enzymes, nucleic acids, receptors, and cells has been used to detect important biological molecules such as antigens, nucleic acids, amino acids, creatinine, urea, and glucose. Enzymes are biological recognition molecules commonly employed in research and development because most chemical reactions in living systems are catalyzed by enzymes. Enzymes are often immobilized on solid substrates so that they can be reused. As a consequence, immobilization strategies for enzymes are of paramount important to preserve their biological activity [12]. Methods for immobilization of enzymes include adsorption, cross-linking, covalent bonding, entrapment and

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encapsulation. The most common methods are adsorption and covalent bonding. Physical adsorption of the enzyme based on van der Waals attractive forces is the oldest and simplest immobilization method. In this case a solution of enzyme, a suspension of cells or a slice of tissue is immobilized by an analyte permeable membrane as a thin film covering the transducer. The adsorption method does not require chemical modification of the biological components and it is possible to regenerate the matrix membrane. The advantage of this method is the simplicity and the great variety of beads that could be used. However, loss of adsorbed enzymes is possible if changes in pH, ionic strength or temperature occur during measurements. Entrapping the enzyme in matrices such as gels, polymers, pastes, or inks considerably improves its stability and consequently the biosensor performance. In addition, it may be essential to covalently link the biological recognizer to the solid support. Covalent bonding may be used to achieve the immobilization of enzymes to a membrane matrix or directly onto the surface of the transducer. These methods are based on the reaction between the same terminal functional groups of the protein (not essential for its catalytic activity) and reactive groups on the solid surface of the insoluble bed. Functional groups available in the enzymes or proteins mainly originate from the side chain of the amino acid. They include, for example, the ε-amino groups from lysine, carboxyl groups from aspartate and glutamate, sulfhydryl groups from cysteine and phenolic hydroxyl groups from tyrosine. Beds as membranes with different active functional groups are able to immobilize enzymes or proteins with great efficiency and facility. Bifunctional reagents (homo or hetero functionals) have also been used in the immobilization of enzymes and or proteins. The method is based on the macroscopic particle formation as a result of the formation of covalent binding between molecules of inert bed with functional reagents. Some of the most used homofunctionals reagents include glutaraldehyde, carbodiimide and others; while the heterofunctionals include 2,4,6-trichloro-1,3,5-triazine, and etc. [13]. It is often that the problem of long-term operational life and storage stability presents crucial hurdles for biosensors' commercial development. The shelf-life of an enzyme-based biosensor usually depends on how long the biological activity of the enzyme can be retained and this may vary from days to months.

A sol–gel matrix has been proved to be a very useful solid support for the immobilization of enzymes as it can retain the enzyme activity and it considered being the best way of immobilizing glucose oxidase (GOx) to date. Several research groups have already employed sol–gel as the enzyme immobilization technique with excellent results [14–17]. Unfortunately, electrochemical-based biosensors are generally not easy to adapt to the sol–gel techniques for the fabrication of their glucose-sensing layer because the sol–gel matrix does not provide good electrical conductivity [17]. Moreover, the procedure of preparing of enzyme-doped silica gel membrane is lengthy and complicated, and difficult to control. Some biomaterials, for instances, silk [18–20], collagen [21,22], and eggshell membrane [23–28] have been employed as platforms for the immobilization of enzymes and the shelf-life of the biosensors were much extended. Eggshell membrane possessing excellent gas- and water-permeability may be an ideal bioplatform for enzyme immobilization. It has been reported that an optical glucose biosensor with eggshell membrane as an enzyme immobilization platform were applied to the determination of glucose content in beverage samples with good results [25]. However, the experimental setup is complicated and requires a bulky and expensive spectrofluorimeter for optical sensing. In addition, most optical biosensors developed so far are not as sensitive as the electrochemical biosensors. They also suffer from the interference from some species in biological samples. This drawback makes the optical biosensor device very complicated in design in order to reduce the effects of interferences. In this article, a simple and relatively cheap electrochemical glucose biosensor consisting of an immobilized enzyme eggshell membrane and a dissolved oxygen electrode is proposed.

Most often glucose oxidase is used to catalyze the oxidation of glucose by oxygen to produce gluconic acid and hydrogen peroxide [29,30].

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D-gluconic acid} + H_2O_2$$

The construction of a glucose biosensor based on the action of GOx offers a suitable approach to determine the glucose content in medicament and drink. This article describes how an eggshell membrane can be employed as a platform for the immobilization of GOx. The shelf-life of the immobilized enzyme is long and the eggshell membrane has excellent permeability to gases and water. This makes it an ideal bioplatform for enzyme immobilization. The proposed glucose biosensor shows good stability and high sensitivity to glucose. It was successfully applied to determine glucose content in real samples and the results were satisfactory.

2. Experimental

2.1. Chemicals and reagents

Glucose oxidase (EC 1.1.3.4. from *Aspergillus niger*) with a specific activity of 245,900 units g^{-1} of solid was obtained from Sigma (St. Louis, MO, USA). β-D-Glucose was from Acros Organics (Geel, Belgium). Glutaraldehyde solution (25%, w/w) in water was purchased from Beijing Chemical Reagent Corporation (Beijing, China). All other reagents were of analytical-reagent grade and used without further purification. The buffer solution for preparing glucose standards was 200 mmol L^{-1} monosodium dihy-

drogen phosphate–disodium hydrogen phosphate solution at pH 7.0. All solutions were prepared with deionized water.

2.2. Instrumentation

Pasco CI-6542 oxygen sensor and Pasco CI-6400 Science Workshop 500 interface were purchased from Pasco Scientific (Roseville, CA, USA). A 81-2 thermostated magnetic stirrer was obtained from Shanghai Kaixin Apparatus Corporation (Shanghai, China). A 721 spectrophotometer was purchased from The Third Analysis Apparatus Corporation of Shanghai (Shanghai, China).

2.3. Immobilization of glucose oxidase on eggshell membrane

An eggshell membrane was carefully peeled from a broken fresh eggshell after the albumen and yolk had been removed. It was cleaned with a copious amount of deionized water. The membrane was placed in a clean watch glass and it was cut into a circle of a diameter of about 15 mm. One-hundred microliters of 0.8% (w/v) GOx solution at pH 7.0 was added. After about 1.5 h, 10 μL of 2.5% (w/w) glutaraldehyde solution as a cross-linking agent was dropped onto the surface of the membrane and left to stand 30 min. A glass rod was gently used to spread the glutaraldehyde solution thoroughly on the membrane surface. The membrane was then immersed in and washed with a pH 7.0 phosphate buffer for 5 min. After washing, the GOx-immobilized eggshell membrane was stored in a pH 7.0 phosphate buffer at 4 °C until further use.

2.4. Assembly of glucose biosensor and determination

The GOx-immobilized eggshell membrane was positioned on the surface of a Pasco CI-6542 oxygen sensor and kept in a steady position by an O-ring. The electrode was immersed into a stirred 10-mL phosphate buffer solution (pH 7.0). Various volumes of standard (0.50 mol L^{-1}) or sample glucose solution were injected into the phosphate buffer with the use of a syringe. The dissolved oxygen signal was captured and processed by a datalogger system consisting of a Science Workshop 500 interface, serial cables, a power supply, and control software. The data were logged in a computer for real-time display and processing.

3. Results and discussion

3.1. Response behavior of glucose biosensor

The oxygen electrode acting as an oxygen transducer was employed to measure the rate of oxygen consumption in the enzymatic oxidation of glucose. The analytical signal of the glucose biosensor is the decrease in the dissolved oxygen content upon exposure to glucose solution. A typical response curve of glucose biosensor is shown in Fig. 1. The decrease in the oxygen level was found to be proportional to the glucose concentration. A linear calibration curve plotting

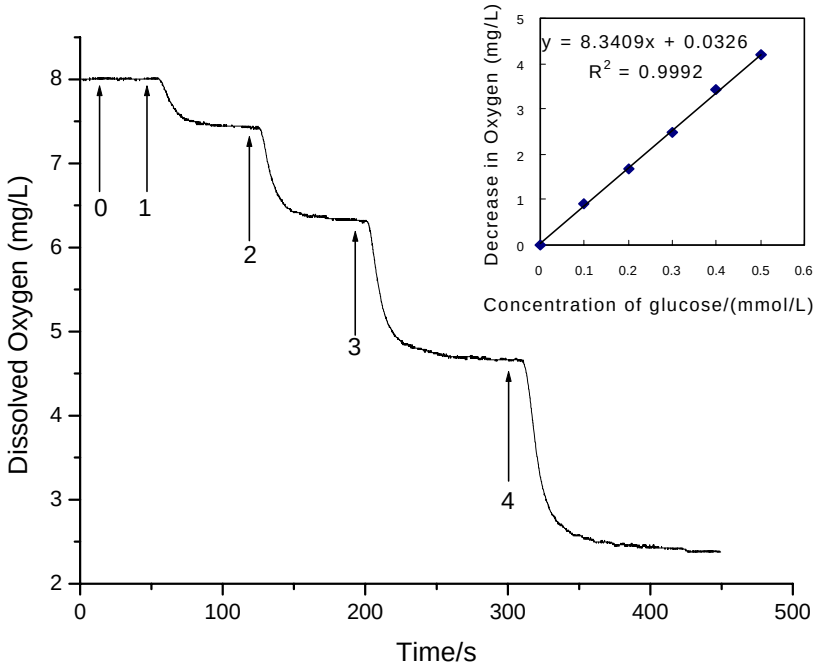

Fig. 1. The dissolved oxygen content of a phosphate buffer (pH 7.0) after each successive addition of various volumes of glucose standard (0.50 mol L^{-1}): (0) 0.0; (1) 2.0; (2) 4.0; (3) 6.0; and (4) 8.0 μL. The inset displays the linear calibration curve of the glucose biosensor.

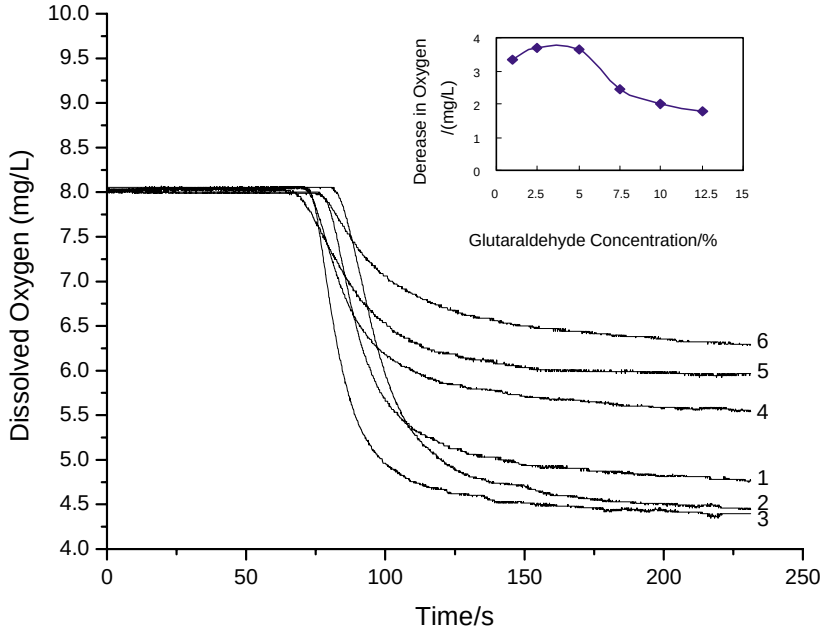

Fig. 2. The dissolved oxygen content of a phosphate buffer (pH 7.0) after addition of same volume of glucose standard (0.50 mol L^{-1}) when the enzyme was cross-linked by various concentrations of glutaraldehyde solution: (1) 1.0; (2) 2.5; (3) 5.0; (4) 7.5; (5) 10; and (6) 12.5% (w/w). The inset displays the relative response of the glucose biosensor against the concentration of glutaraldehyde solution.

the decrease in the oxygen level against the concentration of glucose is shown in the inset in Fig. 1.

3.2. Effect of the concentration of glutaraldehyde as a cross-liking agent

Fig. 2 displays the dissolved oxygen content in a phosphate buffer at pH 7.0 after addition of the same amount of glucose standard (0.50 mol L^{-1}) when the enzyme-immobilized membrane was cross-linked by various concentrations of glutaraldehyde solutions. The results showed that in the range of 1.0–5.0% (w/w) glutaraldehyde the response of the biosensor increased with the increase in glutaraldehyde concentration. But in the range of 5.0–12.5% (w/w) the response of the biosensor decreased apparently. Covalent bonding may be used to achieve the immobilization of biological components to a membrane matrix. These methods are based on the cross-linking reaction between the same terminal functional groups of the protein and reactive groups on the solid surface of the insoluble bed. In our studies, the amino groups of the eggshell membrane and the enzyme can react with glutaraldehyde as shown in Fig. 3.

Since the glucose biosensor is based on the decrease in oxygen level and the consumption of dissolved oxygen strongly depends on the enzymatic activity of the oxidation of glucose, any change in the activity of the enzyme would affect the sensitivity of the glucose biosensor. In general, higher glutaraldehyde concentration would denature most of the enzymes and lead to the decrease in sensitivity of the glucose biosensor. Although the use of 5.0% (w/w) glutaraldehyde solution as cross-linking agent can acquire a little better sensitivity than that of 2.5% (w/w) glutaraldehyde solution, the repeatability of the biosensor is not promising. As a result, 2.5% (w/w) glutaraldehyde solution was chosen as the optimum cross-linking agent for our biosensor.

3.3. Effect of pH

The pH effect was investigated over the range pH 3.0–10.0. Fig. 4 depicts the normalized decrease in oxygen

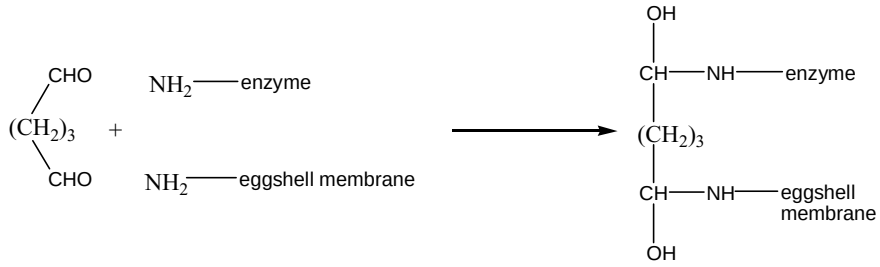

Fig. 3. Reactions of glucose oxidase, glutaraldehyde and protein fiber on eggshell membrane.

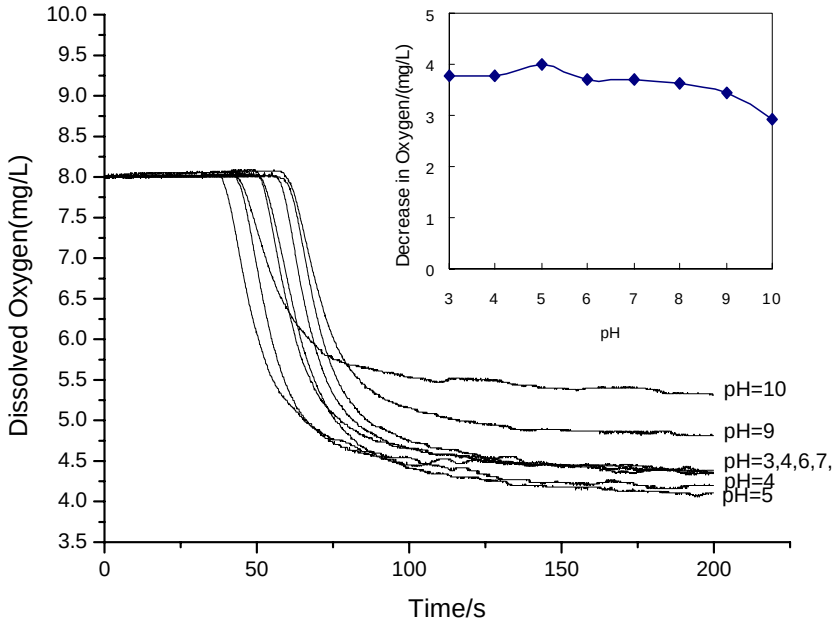

Fig. 4. The dissolved oxygen content of a phosphate buffer after addition of same volume of glucose standard (0.50 mol L^{-1}) at various pH phosphate buffer solutions. The inset displays the relative response of the glucose biosensor against pH.

level of the sensor against pH when the sensor was subject to 0.50 mmol L^{-1} glucose standard at various pH phosphate buffer solutions. The results showed that the response of the glucose biosensor could be maintained above 90% in the pH range of 3.0–8.0. It was found that the change in pH did not apparently affect the dynamic working range of the sensor. The sensor has a very broad pH working range.

3.4. Effect of buffer concentration

Fig. 5 displays the effect of buffer concentration on the dissolved oxygen content in a phosphate buffer at pH 7.0. It is noted that the consumption of the dissolved oxygen increased with the increase in buffer concentration in the range of 1.00–200 mmol L^{-1}. But the response started to decline upon the increase in phosphate concentration when

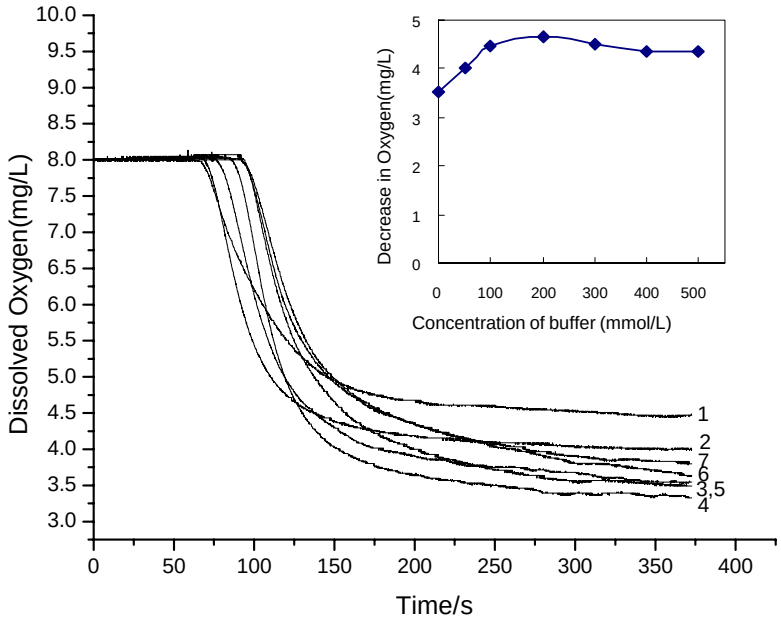

Fig. 5. The dissolved oxygen content of a phosphate buffer (pH 7.0) after addition of same volume of glucose standard at various phosphate buffer concentrations: (1) 1.00; (2) 50.0; (3) 100; (4) 200; (5) 300; (6) 400; and (7) 500 mmol L^{-1}. The inset displays the relative response of the glucose biosensor against the phosphate buffer concentration.

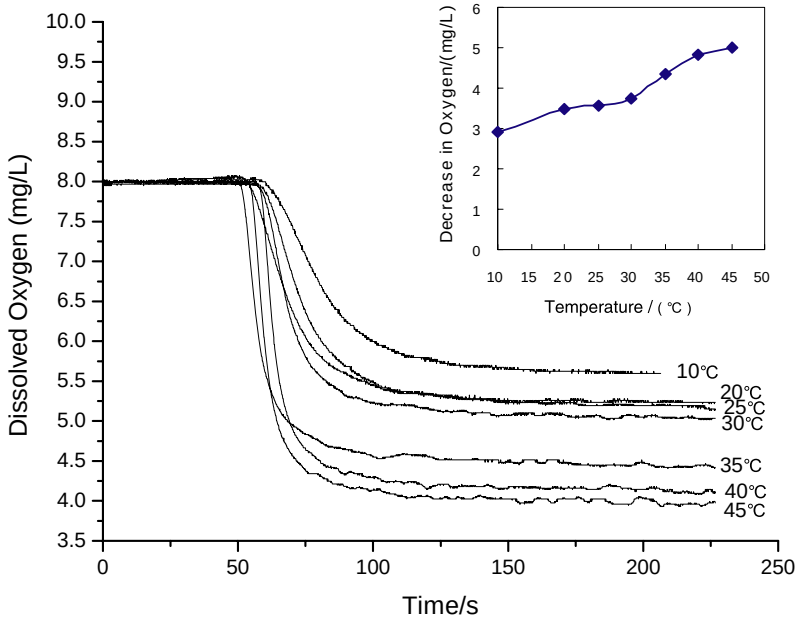

Fig. 6. The dissolved oxygen content of a phosphate buffer (pH 7.0) after addition of same volume of glucose standard at various temperatures. The inset displays the relative response of the glucose biosensor against temperature.

the concentration of phosphate buffer increased to above 200 mmol L^{-1}. As a result, 200 mmol L^{-1} was chosen as the optimal phosphate buffer concentration for our biosensor.

3.5. Effect of temperature

Fig. 6 shows the effect of temperature on the dissolved oxygen content. The consumption of the dissolved oxygen increased with the increase in temperature on exposure to 0.50 mmol L^{-1} glucose. The analytical performance of enzyme-immobilized eggshell membrane is anticipated to be temperature dependent. Higher temperature would result in a drop in lifetime and lead to the decrease in the response of the glucose biosensor. On the other hand, increasing working temperatures has a counterbalance effect on the biosensor. The activity of an immobilized-enzyme is governed by the kinetics of the enzymatic reaction. The reaction rate is faster with increase in working temperature. In our experiment the consumption of the dissolved oxygen increased with the increase in temperature on exposure to 0.50 mmol L^{-1} glucose. The possible reasons are that the enzyme can acquire higher activities at higher temperatures and subsequently consume oxygen at a faster rate in the enzymatic reaction of glucose. Although the response was highest at 45 °C, for practical reasons room temperature is recommended to be used in order to prolong the life-time of the biosensor since most enzymes can easily be denatured at high temperature.

3.6. Analytical figures of merit of glucose biosensor

In this study, the response time was taken as 100 s to record the decrease in the dissolved oxygen level. The linear response of the biosensor for the determination of glucose is 1.0×10^{-5} to 1.3×10^{-3} mol L^{-1}. The precision of the biosensor was also been studied by subjecting the glucose biosensor to 0.50 mmol L^{-1} glucose standard for seven times (R.S.D. = 3.26%, $n = 7$). It confirmed that the biosensor exhibited very desirable analytical features.

3.7. Shelf-life of glucose biosensor

The shelf-life of the biosensor was tested weekly over 4 months at 4 °C. When the biosensor was stored in a refrigerator at 4 °C, the decrease in the oxygen level of the biosensor on exposure to 0.50 mmol L^{-1} glucose was found to be above 85.2% of its initial value over the period. We anticipate that the shelf-life should be much longer as we are still in the progress of monitoring its shelf-life weekly. The excellent shelf-life of the glucose biosensor consisting of GOx immobilized on eggshell membrane is possibly related to the biological compatibility of the eggshell membrane with the enzyme. Eggshell membrane is mainly composed of biological molecules and protein fibers, which may supply polycations

Table 1
Effect of potential interferents on the glucose biosensor

Interferent	Concentration (mmol L^{-1})	Decrease in oxygen level equivalent to glucose (mmol L^{-1})
Citric acid	10	−0.040
Sodium benzoate	50	−0.018
D-Fructose	20	0.138
Sucrose	50	0.054
L-Ascorbic acid	10	0.093
Ethanol	1.0 % (v/v)	−0.014

Table 2
Determination of glucose contents in real samples using the proposed glucose biosensor and commercial glucose kit, and recovery test for wine samples using the glucose biosensor

Sample	Concentration of glucose (mmol L^{-1})		Concentration of glucose added (mmol L^{-1})	Concentration of glucose found[b] (mmol L^{-1})	Recovery (%)	R.S.D. (%)
	Proposed method	Commercial glucose assay kit (spectrophotometric method)[a]				
5% glucose injection preparations	4.75% (w/v)	4.91% (w/v)	–	–	–	–
10% glucose injection preparations	9.75% (w/v)	9.83% (w/v)	–	–	–	–
Changyu dry red wine	12.5	12.1	12.5	11.2	90	5.9
			25	24.8	99.2	2.9
			50	51.6	103.2	2.9
Changyu natural white wine	177	166.8	25	23.5	94	4.0
			125	139	111	2.1
			250	260	104	2.3
Tonghua Natural wine	325.4	329.7	25	26	104	5.8
			125	125	100	1.9
			250	248	99.2	1.9

[a] Reference [31].
[b] Average value from three determinations.

to stabilize the enzyme. The net-veined structure and the gas-permeable hydrophilic property of eggshell membrane can provide an excellent biological micro-environment for the enzyme to survive and maintain its enzymatic activity.

3.8. Interference test

The effect of potential interferents on the decrease in the oxygen level of the glucose biosensor was evaluated by exposing to interferents in 200 mmol L^{-1} phosphate buffer at pH 7.0. The decrease in the oxygen level was calculated as glucose concentration equivalence (i.e. the concentration of glucose that can produce the same decrease as the interferents). The results are displayed in Table 1. It was found that ethanol, D-fructose, citric acid, sodium benzoate, sucrose and L-ascorbic acid only produced slight interference.

3.9. Sample analysis

The proposed biosensor method was employed to determine the glucose content in commercial glucose injection preparations and wines. The glucose contents in the two kinds of glucose injection samples were determined to be 4.75 and 9.75% (w/v), respectively, whereas the glucose contents displayed on the labels were 5 and 10% (w/v), respectively. The analytical performance of the glucose biosensor was satisfactory. The glucose content in the real samples was also determined by our glucose biosensor and a commercial glucose assay kit based on a spectrophotometric method [31]. The results are displayed in Table 2. The findings show that the biosensor agrees quite well with the spectrophotometric method. It was also found that the glucose content in the dry wine was the least among the three wines. Furthermore, the recovery tests for glucose were performed by adding various amounts of glucose to the wine sample solutions. The amounts of added glucose were then evaluated by using the proposed glucose biosensor. The results of the recovery of the samples are summarized in Table 2. The recovery tests demonstrate that the glucose biosensor offers an excellent, accurate and precise method for the determination of glucose in real samples.

4. Conclusion

The glucose biosensor has been successfully applied to determine the concentration of glucose in some commercial products. It also exhibits excellent stability with a long shelf-life of at least 4 months. We are confident that the proposed glucose biosensor can provide a reliable, accurate method for the determination of glucose in real samples. To most important, the promising features of our device are simple sensor design and easy operation in comparison to our previous optical glucose biosensor [25]. Future work directed to the fabrication of other enzyme-based biosensors using eggshell membrane as the immobilization platform is anticipated to be promising.

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