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Article

Multianalyte Biosensor for Simultaneous Determination of Glucose and Galactose Based on Micromachined Chamber-type Electrodes†

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An amperometric multianalyte biosensor for the simultaneous determination of glucose and galactose was developed based on chamber-type electrodes, which were fabricated by micromachining technology. The dual chamber-type enzyme electrode with glucose and galactose sensor elements was integrated onto one microchip. The experimental parameters of this biosensor were optimized. The biosensor exhibited a linearity of up to 4.0 mol/L for glucose and 4.5 mol/L for galactose, and the response time was about 30 s for glucose and 40 s for galactose. No cross-talking behavior was investigated in the course of simultaneous measurement of the two analytes. Interference from electroactive species, such as ascorbic acid and uric acid, was minimized due to the permselectivity of Nafion film. In addition, the biosensor displayed a storage stability of longer than one month.

Keywords multianalyte biosensor, micromachining technology, chamber-type electrode

Introduction

The development and application of multianalyte biosensing devices have become a significant trend in biomedical, biotechnology, industrial and environmental fields.[1-3] Therefore, a great deal of scientific research has been devoted to fabrication of multianalyte biosensing devices suitable for simultaneous measurement of multicomponent.[4-10] Since last decade, the microfabrication techniques have been turned out to be attractive technologies for the chemical sensor and biosensor developments. Using microfabrication techniques, the reduction of the required sample volume of performing multicomponent analysis may be combined with a cost reduction by mass production.

Most of the multianalyte biosensing devices were fabricated so far by using the planar microfabricating processes such as the thin and thick film technologies.[11-19] In the planar microfabricating processes, membrane materials and enzymes were usually integrated as thin layers on top of the planar transducers. In some cases, however, the problems, such as insufficient mechanical strength and inadequate membrane adhesion, may cause the sensor malfunction. In last few years, a micromachining technology has added new impetus to the family of the microfabrication processes in miniaturing the sensors or systems. The technique, including anisotropic etching of silicon, field-assisted bonding along with conventional photolithography, is applied to construct a three dimensional microstructure onto one sensor chip. The chamber-type sensor chip with a three dimensional microstructure provides a protective and controlled environment surrounding the microsensing part. It facilitates the enzyme immobilization, and minimizes the flow effect and chemical interference. Due to its advantage, the chamber-type chemical sensors and biosensors for a single analyte detection fabricated by a micromachining technology have been undertaken by our group and some other researchers.[20-23] Recently, it attracts great interests in developing multianalyte biosensing devices based on micromachining technology.[5,7,24-26] Most of the multianalyte biosensors simultaneously measure the multicomponents by fluorometric or colorimetric method. However, very few papers have been reported about amperometric multianalyte biosensors fabricated by micromachining technology.

In this report, a novel amperometric multianalyte biosensor, dual chamber-type biosensor, has been developed by using a micromachining technology for the

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simultaneous determination of glucose and galactose. Both glucose and galactose are important physiological parameters in clinical analysis. Therefore, these two substances were selected as model analytes to show the general application of this biosensor. The operational conditions of the sensor were optimized. The cross-talk free simultaneous determination of the two analytes using the biosensor was demonstrated. In addition, the analytical characteristics of the dual chamber-type biosensor were investigated.

Experimental

Reagents

Galactose oxidase (GAO) and glucose oxidase (GOD) were obtained from Sigma. Glutaraldehyde (25%) was obtained from Merck. Nafion (5% solution in mixture of low aliphatic alcohols and water) was purchased from Fluka. Galactose, glucose and gelatine were from Shanghai Biochemical Reagent Company (Shanghai, China). All other reagents were of analytical grade. All solutions were prepared with doubly distilled water.

Apparatus and measurements

All electrochemical measurements were conducted using an EG&G potentiostat/galvanostat 273 (Princeton, NJ), and recorded on a LM20A X-Y recorder (Shanghai, China). Ag/AgCl electrode and a platinum foil were used as a reference electrode and a counter electrode, respectively. The current detection and the voltage output were performed with a double potentiostat for the simultaneous determination of glucose and galactose.

The measurements were performed with a three-electrode system in 0.1 mol/L phosphate buffer solution (pH 7.2) with a constant stirring. The background current was allowed to stabilize after the potential was applied. Successive additions of stock glucose or galactose solutions were injected and the current-time data were recorded.

Fabrication of dual chamber-type microchips

The schematic diagram of a double chamber-type microchip is shown in Figure 1. A 2-inch Pyrex glass plate was used as a down-substrate. A 50 nm thick titanium as an intermediate layer and *ca*. 200 nm thick platinum layer were successively deposited on the down-substrate surface by an electron gun. A lift-off technique was used for making a pattern of dual 1.2 mm $\times$1.2 mm Pt working electrodes and conducting line. A 2-inch piece of 300 μm thick p-type silicon (100) wafer with a SiO_2 thin layer on both sides was used as an up-substrate. Two quadratic holes (*ca*. 1.2 mm$\times$1.2 mm) on the SiO_2 layer on one side were obtained by successively using photolithographic and etching processes. Then, silicon was etched anisotropically in 40% KOH solution at 50 ℃ to create a 3-dimensional cavity. The SiO_2 layer was then removed by HF solution. A 400 nm thick silver layer was deposited on the backside of up-substrate. Partial chlorinating of the silver was performed in 0.25 mol/L $FeCl_3$ solution to form an Ag/AgCl reference electrode. The anodic bonding between up-substrate and down-substrate was processed for each chip at 500 ℃ and 300 V for several minutes. The size of an entire chip is 5 mm$\times$5 mm. The chip fabrication was described in more detail elsewhere.[23]

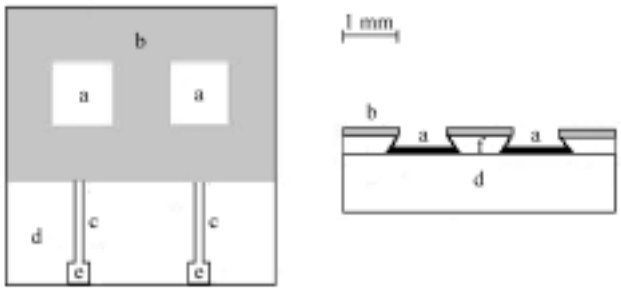

Figure 1 Schematic view of a dual chamber-type microchip. Left: entire view of a microchip; right: cross section of a microchip. (a) Pt working electrodes with Ti intermediate layer; (b) Ag/AgCl reference electrode; (c) conduction line; (d) Pyrex glass; (e) contact pad; (f) silicon layer.

Enzyme immobilization and preparation

10 mg of gelatine was allowed to swell in 0.5 mL of 0.1 mol/L phosphate buffer for 60 min at room temperature and completely dissolved by stirring at 40 ℃ to form a 2% gelatine solution. 1 mg of GOD or GAO was dissolved in 50 μL of warm gelatine solution with stirring to form a homogeneous mixture. 5 μL of the GOD enzyme solution and the GAO enzyme solution were added into the two chambers of the designed microchip respectively, and waited for drying. After gelling, the sensor surface was cleaned and the sensor was examined under a microscope to ensure a properly filled containment. Then, this dual chamber-type electrode was immersed into a 5% glutaraldehyde solution for several minutes to cross-link the enzyme matrix and dried in air. After the deposition of enzyme membrane, 10 μL of Nafion solution (0.5% *V*/*V* in alcohol) was spread several times over chip surface. The sensors were stored in air at 4 ℃ before use.

Results and discussion

Optimization of experimental parameters on the dual chamber-type biosensor

In order to use the dual chamber-type biosensor efficiently, experimental parameters such as operational potential, pH, temperature and buffer solution must be optimized to use the galactose sensor and the glucose sensor on the same chip under a common condition.

The potential dependence of the galactose sensor on the microchip was firstly investigated by a chronoamperometric method in the range of +0.30 V to +0.80 V. The response current increased with potential and reached a maximum value when potential was greater

than 0.7 V. Therefore, a potential of 0.7 V was chosen for the following measurements.

The buffer composition usually has a large impact on the activity of the galactose oxidase. In order to investigate its effect on the chamber-type galactose sensor, several buffer solutions (0.1 mol/L potassium citrate, 0.1 mol/L sodium acetate, and 0.1 mol/L phosphate) were tested in the same galactose substrate. The result showed that the sensitivity was higher in phosphate buffer solution than in others, which was in accordance with our previous report.[27] Therefore, 0.1 mol/L phosphate buffer solution was used for the subsequent study.

The pH dependence of the galactose sensor was determined using a phosphate buffer containing 2 mmol/L galactose as shown in Figure 2. As seen in the figure, the optimum pH was in the range from 7.0 to 7.7. It was reported that an optimum pH for the free galactose oxidase located in the range between 7.0 and 7.3.[28] The results showed that immobilizing enzyme in the chamber had no effect on the enzyme bioactivity. So the optimum pH of 7.2 was selected in the following experiments.

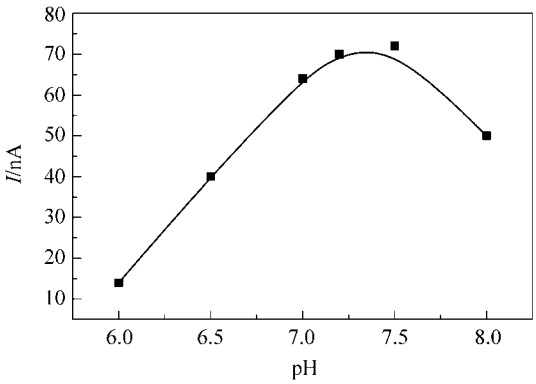

Figure 2 Effect of pH on the galactose biosensor of the dual chamber-type biosensor. The measurements were performed in 0.1 mol/L phosphate buffer (pH=7.2) containing 2.0 mmol/L galactose.

The effect of temperature on the activity of the galactose sensor was investigated in the range of 15 ℃ to 55 ℃. As shown in Figure 3, the response current increased with temperature, and reached a maximum value at 45 ℃. A higher temperature caused a decrease in response current due to a partial enzyme denaturation. In order to maintain the enzyme activity for an extended time, the experiments were tested under room temperature.

A micromachined chamber-type glucose sensor was previously developed by our group.[23] Experiment results showed that its optimum operational conditions of the glucose sensor were similar to those of the galactose sensor. Therefore, the simultaneous measurements for glucose and galactose by the designed dual chamber-type biosensor were performed under the above common conditions (operational potential, pH, temperature, and buffer solution).

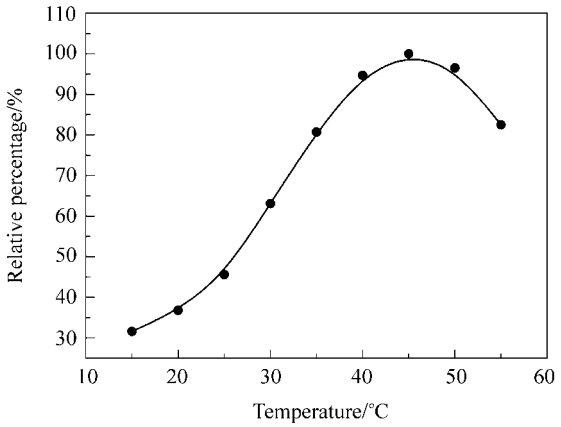

Figure 3 Effect of temperature on the galactose biosensor of the dual chamber-type biosensor. Other conditions are the same as those in Figure 2.

Simultaneous determination of glucose and galactose

A GOD based glucose sensor and a GAO based galactose sensor were integrated onto one microchip for simultaneous measurement of glucose and galactose. The results are shown in Figure 4. The calibration curves of the designed biosensor were obtained by simultaneously measuring glucose and galactose in a 0.1 mol/L phosphate buffer solution (pH=7.2). It showed that the biosensor exhibited a linearity of up to 4.0 mol/L for glucose and 4.5 mol/L for galactose. The sensitivities were 400 nA/(mmol•L^{-1}) for glucose and 36 nA/(mmol•L^{-1}) for galactose. The response time was about 30 s for glucose and 40 s for galactose.

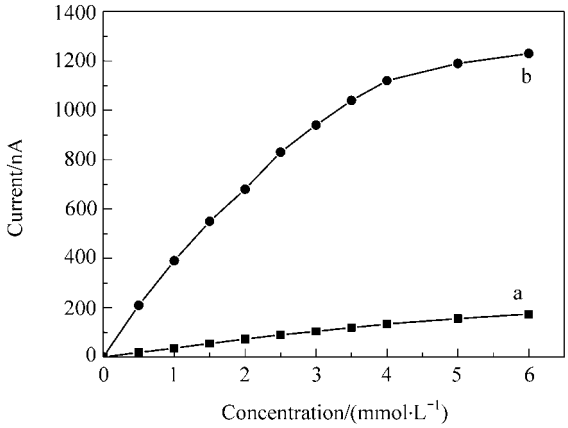

Figure 4 Calibration curves of the dual chamber-type biosensor for the simultaneous measurement of galactose (a) and glucose (b).

Cross-talking behavior of the dual chamber-type biosensor

The enzymatic oxidation of the analytes glucose and galactose produces hydrogen peroxide within the en-

zyme membranes of the sensors. In the course of simultaneous measurements of multianalytes, cross-talking effect may occur between the different sensors resulting from the emission of hydrogen peroxide from one sensor and its correspondent detection from another sensor.

The cross-talking behavior of the designed dual chamber-type biosensor was investigated. In the first experiment, glucose and galactose were added alternately in order to check the response of the glucose sensor on galactose and vice versa. The results are given in Figure 5. No response was observed either at the galactose sensor when glucose was injected (Figure 5a), or at the glucose sensor when galactose was injected (Figure 5b).

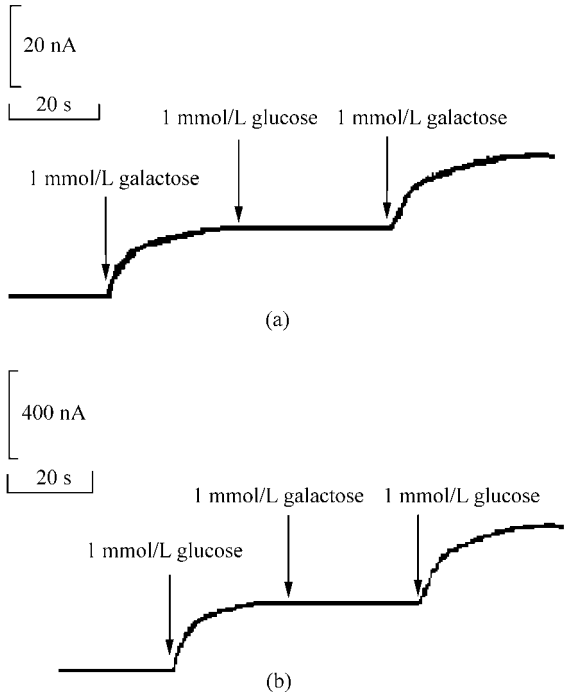

Figure 5 Response of the dual chamber-type biosensor on alternatively added amounts of glucose and galactose. (a) Response of the galactose sensor; (b) response of the glucose sensor.

In order to examine further whether the dual chamber-type biosensor is completely free from cross-talking problems, a second experiment was conducted. As shown in Figure 6, for the measurement by the glucose sensor on the dual chamber biosensor, the calibration curves were taken from solutions containing (1) only glucose, (2) glucose+galactose (1∶1 in molar ratio). The calibration curves were compared between different concentrations of pure glucose solution and mixture solution of galactose and glucose. The response current values for mixture solution of galactose and glucose were almost equal to those obtained by only adding glucose. It means that galactose had no influence on the glucose determination on the dual chamber-type biosensor. Similarly, for the measurement by the galactose sensor, calibration curves were taken in solutions containing (1) only galactose, (2) galactose+glucose (1∶1 in molar ratio). As seen in Figure 7, glucose had no effect on the galactose sensor on the dual chamber-type biosensor.

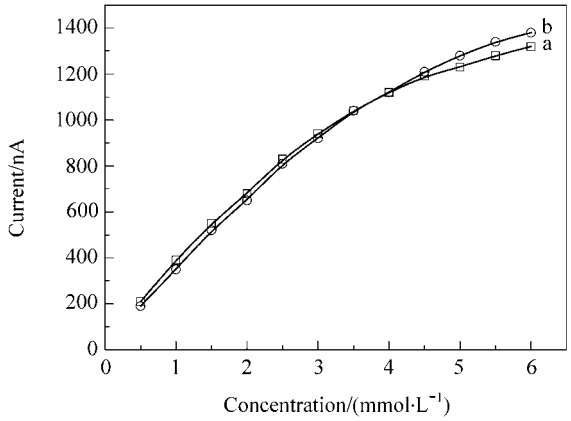

Figure 6 Response of the glucose sensor on the dual chamber-type biosensor. (a) Pure glucose solution; (b) mixture solution of galactose and glucose (1∶1 in molar ratio, noted as glucose+galactose).

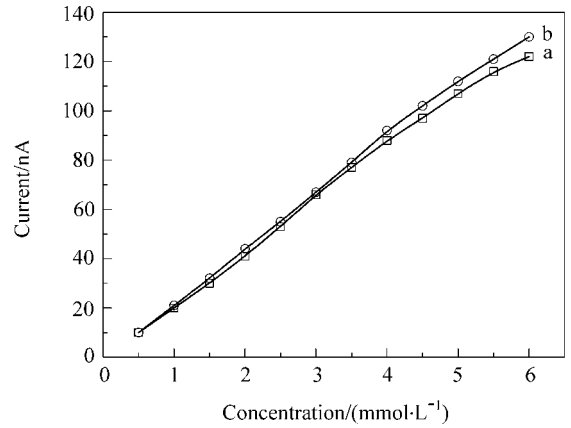

Figure 7 Response of the galactose sensor on the dual chamber-type biosensor. (a) Pure galactose solution; (b) mixture solution of galactose and glucose (1∶1 in molar ratio, noted as galactose+glucose).

From the above results, it could be concluded that the designed biosensor is free from the cross-talking effect. It is attributed to the special three-dimensional chamber-type structure of designed biosensor and the geometrical arrangement of the sensors.[7,25]

Selectivity, reproducibility and stability

In addition to its free cross-talking effect, the dual chamber-type biosensor displayed higher selectivity in the simultaneous determination of glucose and galactose than several other electroactive substances (ascorbic acid and uric acid). The experimental results showed that ascorbic and uric acid did not cause any observable interference. The Nafion film on the biosensor minimized interference from other electroactive species.

The reproducibility was tested for its response to 0.5 mmol/L of glucose and galactose. The relative standard deviation obtained for six successive measurements was around 2% for glucose and 3.1% for galactose.

The stability of the biosensor was also examined. The obtained results showed that no obvious change for the sensor response was observed after continuous experiments for half a month. The designed biosensor was stored in refrigerator at 4 ℃. It was observed that this designed biosensor could be stored over one month with only a slow decline for its performance. It can be seen that the biosensor has good reproducibility and high stability due to its special sensor construction.

Conclusions

A dual chamber-type biosensor for the simultaneous determination of glucose and galactose has been constructed by using a micromachining technology. The dual chamber biosensor exhibited good analytical performances. Furthermore, the biosensor showed free crossing-talking behavior in the course of simultaneous determination of the two analytes. It could be concluded that micromachining technology holds great promise on the fabrication of multianalyte biosensors for simultaneous monitoring of multianalytes.

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