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Amperometric glucose biosensor with remarkable acid stability based on glucose oxidase entrapped in colloidal gold-modified carbon ionic liquid electrode

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ABSTRACT

A colloidal gold-modified carbon ionic liquid electrode was constructed by mixing colloidal gold-modified graphite powder with a solid room temperature ionic liquid n-octyl-pyridinium hexafluorophosphate ($OPPF_6$). Glucose oxidase (GOD) was entrapped in this composite matrix and maintained its bioactivity well and displayed excellent stability. The effect conditions of pH, applied potential and GOD loading were examined. Especially, the glucose oxidase entrapped in this carbon ionic liquid electrode fully retained its activity upon stressing in strongly acidic conditions (pH 2.0) for over one hour. The proposed biosensor responds to glucose linearly over concentration range of 5.0×10^{-6} to 1.2×10^{-3} and 2.6×10^{-3} to 1.3×10^{-2} M, and the detection limit is 3.5×10^{-6} M. The response time of the biosensor is fast (within 10 s), and the life time is over two months. The effects of electroactive interferents, such as ascorbic acid, uric acid, can be significantly reduced by a Nafion film casting on the surface of resulting biosensor.

1. Introduction

Glucose biosensors are the most widely studied because of their importance in the monitor of blood glucose for the treatment and control of diabetes. Most of the amperometric glucose biosensors are based on the glucose oxidase (GOD), GOD contains two tightly bound flavine adenine dinucleotide (FAD) cofactors and can catalyze the electron transfer from glucose to oxygen with an accompanying production of gluconolactone and hydrogen peroxide. The following reactions display the transducing principle (Kenausis et al., 1997; Berchmans et al., 2002):

$$\text{GOD–FAD} + \text{glucose} \rightarrow \text{GOD–FADH}_2 + \text{gluconolactone} \quad (1)$$

$$\text{GOD–FADH}_2 + \text{O}_2 \rightarrow \text{GOD–FAD} + \text{H}_2\text{O}_2 \quad (2)$$

The quantitation of glucose can be achieved via electrochemical detection of the enzymatically liberated H_2O_2.

Ionic liquids (ILs), as molten salts with the melting point close to or below room temperature, consist of two asymmetrical ions of opposite charges (organic cations and various anions) that only loosely fit together. Compared to commonly used electrolyte solvents, ILs are reported to have a wide potential window, good electrical conductivity, high ionic mobility, excellent chemical and thermal stability, negligible vapor pressure, a wide liquid range, low combustibility, and the ability to solvate a diverse range of compounds. Because of these favorable properties, ILs are increasingly attracting the attention in various areas of electrochemistry (Wei and Ivaska, 2008).

Carbon paste electrodes (CPEs), first introduced by Adams (1958), consist of a mixture of an appropriate ratio of graphite powder and a non-conductive liquid binder such as mineral oil, paraffin or Nujol. Because of numerous advantageous properties such as low cost, low background current, relatively wide potential window, ease of fabrication, high sensitivity, renewable surface and tunable composition, these electrodes are widely used in different electrochemical applications (Svancara and Schachl, 1999; Kalcher et al., 1995). Recently, CPEs made by using ILs as a binder instead of non-conductivity binder for analytical purposes, called carbon ionic liquid electrode (CILE), have been reported in a few studies (Liu et al., 2005; Maleki et al., 2006; Safavi et al., 2006; Shul et al., 2006). CILE was utilized for analysis of different species such as nitrite, NADH, dopamine, ascorbic acid, and uric acid in those reports. Due to thermal stability and high conductivity of ILs, it would be advantageous to use ILs to fabricate IL-carbon paste biosensors. A number of studies have shown that biosensors can be operated in ILs instead of aqueous medium which results in increasing the life time of the biosensor (Xiong et al., 2007; Wang et al., 2007). Modifying the electrode surface with a thin film of an ionic liquid and then immobilizing the enzyme of choice on that film was also shown to be

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useful in preparing biosensors for several applications (Du et al., 2007; Zhao et al., 2007; Sun et al., 2007). Lately, "a solid ionic liquid at room temperature" was used to prepare attractive composite electrodes for amperometric biosensing (Musameha et al., 2008).

Colloidal gold is an extensively used metal colloid, which has been used for study of direct electrochemistry of proteins (Brown et al., 1996; Gu et al., 2001; Liu and Ju, 2003). It provides an environment similar to that of redox proteins in native systems and gives the protein molecules more freedom in orientation, thus reducing the insulating property of the protein shell for the direct electron transfer and facilitating the electron transfer through the conducting tunnels of colloidal gold.

In this report, a gold colloidal modified carbon ionic liquid composite glucose biosensor using gold colloidal modified graphite powder mixed with n-octyl-pyridinium hexafluorophosphate ($OPPF_6$) and GOD was fabricated for the detection of glucose. The electrode combined many advantages of CPE, ILs and colloidal gold, such as, ease of fabrication, renewable surface and tunable composition of CPE, good electrical conductivity and excellent chemical stability of ILs, good biocompatibility and excellent electron transfer capability of colloidal gold. The effect of experimental conditions and performance of this resulting biosensor were investigated in detail. In particular, the excellent acid stability of the biosensor was examined.

2. Experimental

2.1. Reagents and apparatus

GOD (EC 1.1.3.4, type II from *Aspergillus niger*, activity: 112 U mg^{-1}) was purchased from Amresco and used as received. D-(+)-glucose, $AuC1_3HCl{\cdot}4H_2O$ (Au% > 47.8%) and Nafion (5 wt%) was obtained from Sigma–Aldrich. Graphite powder (extra pure, from Sinopharm Chemical Reagent Co., Ltd., Shanghai), paraffin oils (from Aladdin Reagent Co., Ltd., China) and ionic liquid $OPPF_6$ (from Shanghai Chengjie Chemical Co., Ltd., China) were used for the preparation of traditional CPE and CILE. All other chemicals were of analytical grade and used without further purification. Colloidal gold was prepared according to literature (Doron et al., 1995) by adding sodium citrate solution to a boiling $HAuCl_4$ aqueous solution. The diameter of colloidal gold nanoparticles was measured by TEM (Supplemental Fig. S.1). The TEM image showed gold nanoparticles with about 15 nm in diameter. 0.1 M phosphate buffer solutions (PBS) with various pH values were prepared by mixing stock standard solutions of Na_2HPO_4 and KH_2PO_4 and adjusting the pH with 0.1 M H_3PO_4 or NaOH. All solutions were made up with twice-distilled water.

All electrochemical experiments were performed with a CHI660A electrochemical workstation (Shanghai Chenhua Apparatus Co., China) in conjunction with a standard three-electrode system: a glucose oxidase-colloidal gold-modified carbon ionic liquid enzyme electrode (3 mm in diameter) was used as working electrode; a platinum wire was applied as the counter electrode and an Ag/AgCl (3 M KCl) reference electrode. All potentials were reported with respect to the reference electrode. TEM image was obtained with a JEM-3010 transmission electronic microscope.

2.2. Preparation of the glucose biosensor

The colloidal gold-modified carbon ionic liquid electrode (Au-CILE) was prepared according to the following procedure: Prior to use, the graphite powder was heated to 700 °C for 30 s in a muffle furnace in order to remove possible amorphous carbon impurities. Then the graphite powder was cooled to room temperature in a desiccator. 50 mg of pretreated graphite powder was mixed thoroughly with 150 μL colloidal gold solution. After evaporation of water in a desiccator, 50 mg $OPPF_6$ was added to the mixture and mixed in an agate mortar. The GOD–Au-CILE was prepared by adding 50 mg $OPPF_6$ and 2.5 mg GOD to the dried mixture of colloidal gold-modified carbon graphite powder. As a comparison, 2.5 mg GOD, 50 mg carbon graphite powder were mixed thoroughly with 50 mg $OPPF_6$ for preparation of GOD–CILE. A portion of the resulting composites was put into teflon tubes with the inner diameter of 3 mm to form different electrodes (Au-CILE, GOD–Au-CILE and GOD–CILE). Electrical contact to the pastes was established by a copper wire down the teflon tube and into the back of the mixture. After the electrode tips were smoothed manually with a clean paper and then with a weighing paper to produce the flat surface. Finally, 5 μL 0.5% Nafion solution was cast on the surface of the prepared biosensor, following experiments were carried out. The prepared electrodes were stored in refrigerator (4 °C), when not in use.

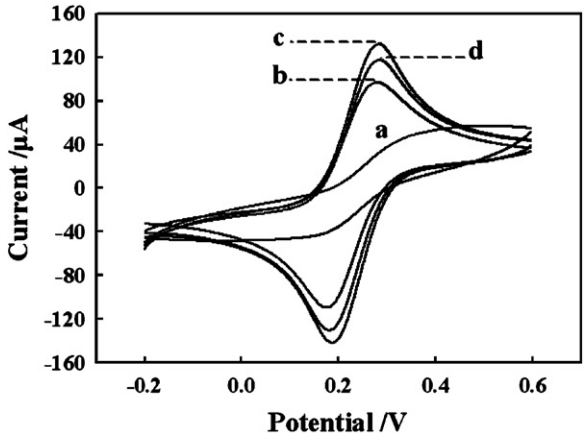

Fig. 1. Cyclic voltammograms for 5 mM potassium ferricyanide using GOD–CPE (a), GOD–CILE (b), Au-CILE (c), GOD–Au-CILE (d), respectively. scan rate 50 mV s^{-1}.

3. Results and discussion

3.1. Electrochemical characterization of enzyme electrodes

Cyclic voltammetric measurements were performed in a 5 mM $[Fe(CN)_6]^{3-/4-}$ probe solution containing 0.1 M KCl. As shown in Fig. 1, compared with the GOD–CPE (Fig. 1a), a pair of well-defined redox peaks of $[Fe(CN)_6]^{3-/4-}$ probe was observed on the GOD–CILE (Fig. 1b), Au-CILE (Fig. 1c), GOD–Au-CILE (Fig. 1d), because of good electrocatalytic activity and conductibility of the ionic liquid ($OPPF_6$). Additionally, due to good conductibility of colloidal gold nanoparticles, the peak current at Au–GOD–CILE was much larger than that obtained at GOD–CILE. Compared with Au-CILE, a remarkable decrease of peak current was obtained at GOD–Au-CILE, suggesting that the bulky GOD molecules blocked the electron exchange between the redox probe and electrode surface.

3.2. Optimization of experimental conditions

3.2.1. Optimization of solution pH

The glucose biosensor exhibited good current response in 0.1 M phosphate buffer solution in the pH range from 5.0 to 8.0. As showed in Supplemental Fig. S.2, the response increased above and decreased below the pH range. However, the biosensor showed maximum response at pH 7.0, which was chosen for subsequent experiments.

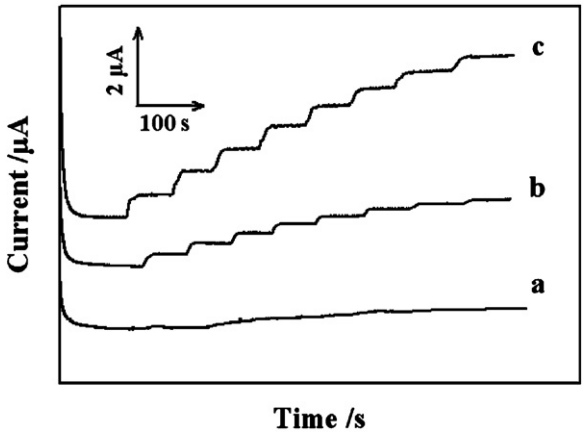

Fig. 2. Current–time recordings for successive additions of 0.1 mM glucose at GOD–CPE (a), GOD–CILE (b), GOD–Au-CILE (c), measured at +0.8 V.

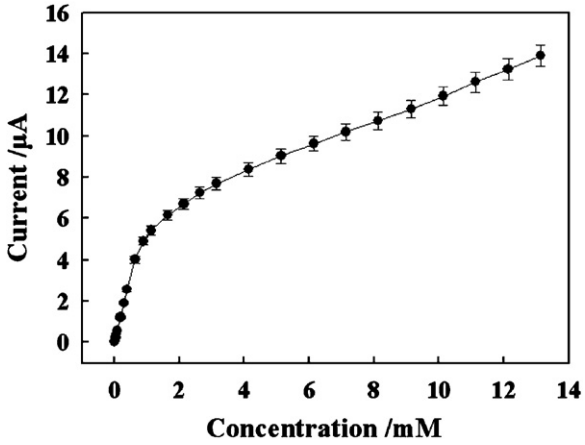

Fig. 3. The calibration curve of the response current at GOD–Au-CILE to glucose concentration in 0.1 M PBS (pH 7.0), measured at +0.8 V.

3.2.2. Optimization of applied potential

To improve the performance of the biosensor, the effect of applied potential on the response of proposed biosensor to glucose had been investigated in detail. The effect of applied potential on the response current is shown in Supplemental Fig. S.3. With the increase of the applied potential from 0.5 to 1.0 V, the response current increased accordingly. This meant that the response of the enzyme electrode is resulted from the electrochemical oxidation of hydrogen peroxide. When the applied potential was higher than 0.8 V, the response current began to level off and the noise which was estimated as the fluctuating value of the steady-state current increased obviously. Thus, a potential of 0.8 V was selected for glucose detection. At this potential, a higher sensitivity and a good signal/noise ratio could be offered.

3.2.3. Optimization of GOD loading

Optimization of the enzyme loading was necessary to improve the measured response. Supplemental Fig. S.4 assesses the effect of GOD loading on the steady-state response current of the biosensor to 0.25 mM glucose in 0.1 M PBS (pH 7.0), the applied potential was +0.8 V. The maximum response was showed at 2.5 mg of GOD loading, which was chosen for subsequent experiments.

3.3. Amperometry of glucose

The electrocatalytic activity of the GOD–CPE, GOD–CILE and GOD–Au-CILE were investigated by steady-state current–time response curves to the successively addition of 0.1 M glucose at an applied potential of +0.8 V in 0.1 M PBS (pH 7.0), which is shown in Fig. 2. It is clear that response to glucose at GOD–CPE was very small and slow. However, rapid (within 10 s) and sensitive response to glucose could be achieved for GOD–CILE and GOD–Au-CILE. And interestingly, the amperometric response signal of GOD–Au-CILE is much greater than that of GOD–CILE, which would be because of good biocompatibility and excellent electron transfer capability of colloidal gold nanoparticles.

The current–time response of the biosensor upon the successive injection of glucose into electrochemical cell with stirring was examined. The oxidation current increased steeply and then reached over 95% of the steady-state current in less than 10 s. The result meant that the response of this biosensor was very fast. Fig. 3 shows that the linear response range of the biosensor to glucose concentration was from 5.0×10^{-6} to 1.2×10^{-3} and 2.6×10^{-3} to 1.3×10^{-2} M with correlation coefficients of 0.9987 and 0.9992. The regression equation was i (μA) = $3.71 \times 10^{-3} + 6.39C_{glucose}$ (mM) and i (μA) = $5.83 + 0.61C_{glucose}$ (mM). The detection limit was estimated to be 3.5×10^{-6} M defined from a signal/noise ratio of 3.

The apparent Michaelis–Menten constant (K_m^{app}) can be calculated from the electrochemical version of the Linweaver–Burkequation (Kamin and Wilson, 1980):

$$\frac{1}{i_{ss}} = \frac{1}{i_{max}} + \frac{K_m^{app}}{i_{max}c}$$

where i_{ss} is the steady-state current after the addition of substrate, i_{max} is the maximum current under saturated substrate conditions, c is the concentration of substrate. The K_m^{app} value for the GOD–Au-CILE electrode was found to be 4.1 mM, which was smaller than the biosensor reported before (Wu et al., 2007; Zeng et al., 2009). The smaller K_m^{app} value meant that GOD entrapped in the Au-CILE exhibited high enzymatic affinity to glucose.

3.4. Acid stability study of the glucose biosensor

Acid stability can be used to measure enzyme's survival under conditions of extreme pH. An acid-stable hydrogen peroxide biosensor based on the immobilization of soybean peroxidase within a self-gelatinizable hydrogel was reported (Wang et al., 1999, 2001). Entrapping enzymes in surfactant-modified silica sol–gel matrixes resulted in a high degree of protection in strongly acidic condition was also reported (Frenkel-Mullerad et al., 2005). Wang's group (2006) reported acid stability of carbon paste enzyme electrodes against harsh pH conditions.

In this report, acid stability was also examined with the resulting glucose oxidase electrode. Fig. 4 displays the influence of the immersion time (in acidic media pH 2.5) upon the response of the GOD enzyme electrode. The resulting biosensor exhibited high stability in connection with the different immersion times, almost retaining their entire original response after a 60-min acid incubation. Fig. 5 shows a study of acid deactivation of GOD entrapped in the Au-CILE by dipping in acidic solution of different pH values. This glucose biosensor of GOD in the Au-carbon ionic liquid matrix retained its complete response after dipping in acidic solutions of pH higher than 2.0. Then, a rapid decay of its current signal (to 45% of its original value) was observed between pH 2.0 and 0.8, with a slower decrease at lower pH values.

The exact reason for the protection of GOD entrapped in Au-carbon ionic liquid matrix against harsh pH condition is not fully understood. But we could now offer some interpretations for this. On one side, immobilized GOD molecules in Au-carbon ionic liquid matrix have restricted mobility, which prevented structural changes and "locked" the enzyme in a certain preferred orientation.

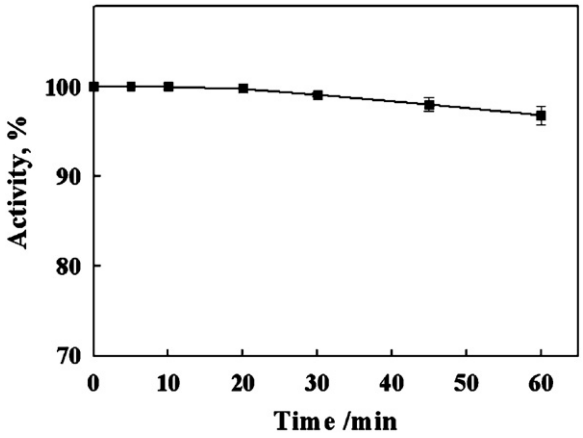

Fig. 4. Acid deactivation kinetics at pH 2.0 of GOD within Au-CILE matrix. The activity was recorded at times indicated using amperometric monitoring of 0.25 mM glucose, applied potential, +0.8 V.

On the other hand, the hydrophobic Au-carbon ionic liquid matrix can play a role as a barrier to hydronium ions. This would lead to considerable insensitivity of the local pH within the microenvironment of GOD–Au-CILE, which can prevent the enzyme's ionogenic groups from changing their ionization state. Similar acid protection were introduced by other researchers (Gole et al., 2001; Frenkel-Mullerad et al., 2005; Wang et al., 2006).

3.5. Reproducibility and stability

The reproducibility and stability of the resulting biosensor have also been studied. The relative standard deviation (RSD) of the current response of GOD–Au-CLIE to 0.25 mM glucose was about 3.9% for 10 successive measurements. For five electrodes modified identically, the R.S.D. was within 5%. The stability of the proposed biosensor under storage conditions (0.1 M PBS, pH 7.0, 4 °C) was investigated. The current response for 0.25 mM glucose was measured every two days up to two months, which did not change in a noticeable way during the first week. After two months, the current response still remained above 85% of its initial response.

3.6. Anti-interference ability and real sample study

Anti-interference ability is important in practical use of the biosensors. The oxidizable compounds such as ascorbic acid (AA) and uric acid (UA) are usually co-existed with glucose in real samples. The current response was obtained in the mixture of glucose and the interfering species at approximate physiological normal levels (2.0 mM glucose, 0.1 mM AA, 0.5 mM UA). The satisfactory anti-interference ability could be resulted from the good properties of the outer Nafion film, which can effectively inhibit the penetration of negatively charged substrates towards the electrode surface (Mao et al., 2000; Shankaran et al., 2003). As shown in Supplemental Fig. S.4, both these interferences have no influence on glucose determination.

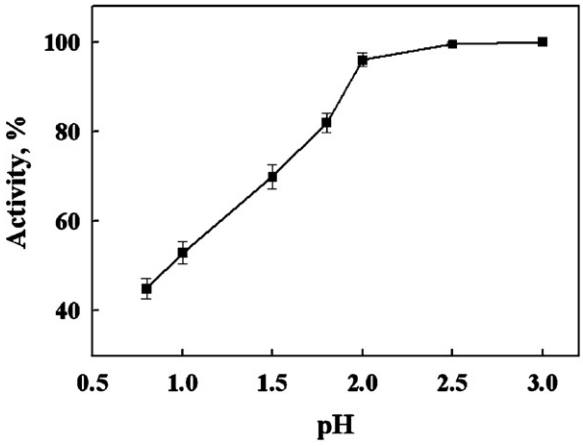

Fig. 5. Influence of the pH upon the enzymatic activity of GOD–Au-CILE. The activity was examined after a 10-min dipping in the indicated pH medium, followed by measurements in 0.1 M PBS (pH 7.0), applied potential, +0.8 V.

Table 1
Results of the glucose assay for human serum samples.

Sample number	Glucose content provided by the local hospital (mM)	Measured by the proposed biosensor (mM)[a]	Relative error (%)
1	4.27	4.48 ± 0.09	+4.9
2	5.34	5.53 ± 0.12	+3.6
3	5.83	5.92 ± 0.16	+1.5
4	6.28	6.15 ± 0.18	−2.1

[a] Average of five measurements ± standard deviation.

Finally, Human serum samples were assayed to demonstrate the practical use of the proposed biosensor. As shown in Table 1, the results obtained by the biosensor were satisfactory and in good agreement with those measured by the biochemical analyzer in the local hospital. Additionally, to demonstrate the practical use of the biosensor in acid media, a real-life sample (Coca-Cola beverage), which has a pH of 2.5, was also assayed. The concentration of glucose determined by this biosensor was found to be 0.33 ± 0.01 mM in this beverage sample, which agree well with the result obtained by HPLC with refractive index detector (0.35 mM).

4. Conclusion

In summary, GOD was successful immobilized in a colloidal gold-modified carbon ionic liquid electrode, which displayed great catalytic ability to glucose. An unusually high level of protection of GOD against harsh pH conditions within a colloidal gold-modified carbon ionic liquid composite matrix was demonstrated. GOD maintained its complete activity following prolonged immersions in highly acidic solutions. The proposed glucose biosensor exhibited high sensitivity and stability; good reproducibility and anti-interference ability, especially excellent acid stability. The unique acid stability of this biosensor indicated great promise for sensing applications as well as for numerous industrial and biotechnological operations involving harsh acidic conditions.

Acknowledgments

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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.2010.04.045.

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