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ORIGINAL PAPER

Enhanced direct electron transfer of glucose oxidase based on a protic ionic liquid modified electrode and its biosensing application

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Abstract A novel glucose oxidase (GOD) biosensor was fabricated with a protic ionic liquid (PIL) *N*-ethylimidazolium trifluoromethanesulfonate ([EIm][TfO]) as the modifier of a carbon electrode. Due to the excellent conductivity and the conformational changes of the microenvironment around the GOD, the electrochemical and biocatalytic properties of GOD immobilized on the PIL-based electrode were dramatically enhanced. A couple of well-defined redox peaks could be observed, with a formal potential of −0.476 V. The GOD biosensor presented good catalytic activity to the oxidation of glucose in oxygen-saturated phosphate buffer solutions. The cathodic peak currents of GOD decreased along with glucose concentrations. A linear response in the range 0.005–2.8 mM was obtained with a detection limit of 2.5 μM. The sensitivity and the apparent Michaelis–Menten constant (K_m) were estimated to be 14.96 μA mM^{-1} and 1.53 μM, respectively. In addition, the biosensor remained stable over 30 days, indicating its good chemical and mechanical stability. The glucose content of several serum samples was determined by using the newly developed biosensor, and the results were in good agreement with those obtained by hospital measurements. All results suggested that PILs were a good media for supporting biocatalytic processes on the bioelectrode.

Keywords Glucose oxidase · Protic ionic liquid · Glucose · Biosensor · Direct electron transfer

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Introduction

Biosensors are attractive in analytical applications due to their unique specificity. It is well-known that glucose biosensors have successfully used glucose oxidase (GOD) to monitor glucose on account of its catalysis of the electron transfer from glucose to oxygen resulting in the production of gluconic acid and hydrogen peroxide [1–5]. GOD, therefore, is an ideal model enzyme in the fields of bioelectrochemical and biosensor research. GOD comprises two identical polypeptide chains; each contains a flavin adenine dinucleotide (FAD) redox center. However, it is rather difficult for GOD to exhibit any voltammetric response on conventional solid electrodes because the active redox center of GOD, FAD is deeply embedded in a protective protein shell. Therefore, it is desirable to develop novel and convenient methods to improve the direct electron transfer (DET) between GOD and electrode. Recently, a variety of attempts have been made to improve the electron transfer between the active redox center and the electrode and to obtain the biosensor with good performance. For example, Oztekin et al. based on the immobilization of GOD on the PPMH (poly 1,10-phenanthroline monohydrate)-modified GC electrode, the direct electrochemistry of GOD was carried out and a glucose biosensor with improved performance was constructed [6]. A one-step enzyme immobilization process with silica sol–gel/polyvinyl alcohol

hybrid film was described by Zuo et al. [7] to achieve direct electrochemistry of GOD on screen-printed electrode. Gao and Zheng [8] studied the direct electron transfer of adsorbed GOD at carbon nanotube/gold nanoparticle/amine-terminated ionic liquid nanocomposite. Direct electrochemistry of the GOD immobilized on a composite matrix based on chitosan and $NdPO_4$ nanoparticles underlying on GC electrode [9]. In most cases, the direct electrochemistry of GOD was carried out based on the synergic effect of the polymer film [10], nanoscale materials (carbon nanotubes [11, 12], gold nanoparticles [13], metal oxide nanoparticles [14]), and other biocompatible materials [15, 16].

Ionic liquids (ILs) are molten salts with the melting point close to or below room temperature. They are generally formed by a large organic cation and an organic or inorganic anion. ILs have many unique chemical and physical properties, such as high chemical and thermal stability, good ionic conductivity, negligible vapor pressure and wide electrochemical windows. Recently, due to their high electrochemical stability and ionic conductivity, ILs have been intensively adopted in the field of electrochemistry and electroanalysis [17–19]. Studies have demonstrated that ILs can be entrapped in conventional matrices, such as chitosan [20, 21], cellulose [22], carbon materials [16, 23, 24], and sol–gel-based silica matrices [25] to construct the biosensor. The resulted composites created a unique kind of material for the immobilization of enzymes. It has been shown that the incorporation of ILs can increase sensitivity and facilitate the DET reaction between proteins and electrode surface [18]. In this respect, attention has been paid mostly to aprotic ILs, especially imidazolium salts [18–20, 26, 27]. However, limited work has been devoted to protic ILs. Protic ILs (PILs), consisting of combinations of Brønsted acids and bases, demonstrate high proton conductivity, and excellent ability to form hydrogen bonds [28, 29]. Persson and Bornscheuer assumed that the hydrogen bond and the electrostatic interaction between the IL and the enzyme resulted in a high kinetic barrier for unfolding of the enzyme, therefore preventing the rigid structure of the enzyme can be protected from being destroyed [30]. Accordingly, the stability and sensitivity of biosensors were improved effectively by the incorporating of PILs into conventional matrices.

Here, a PIL, *N*-ethylimidazolium trifluoromethanesulfonate ([EIm][TfO]), was chosen as an effective modifier for the fabrication of a novel carbon ionic liquid electrode (CILE). The direct electrochemistry and electrocatalytic behavior of GOD on the CILE was investigated. The protic IL [EIm][TfO] was used to retain the bioactivity of GOD and facilitate the direct electron transfer between the GOD and the electrode. The immobilized GOD exhibited excellent electrocatalytic activity for the oxidation of glucose.

Experimental

Reagents

Glucose oxidase (EC 1.1.3.4, 147 U/mg) and Nafion solution were purchased from Sigma. Glucose, paraffin oil (mixture of C_{10}–C_{18} n-alkane) and high purity graphite powder (SP, 2–10 μm in diameter) were obtained from Sinopharm Chemical Reagent Co. Ltd. A stock solution of glucose was prepared and allowed to mutarotate at room temperature overnight before use. [EIm][TfO] was synthesized and purified as reported previously [28, 31]. All other chemicals were of analytical grade and were used without further purification. Double-distilled water was used throughout the work.

Apparatus

Electrochemical characterizations were carried out on a CHI 660 C electrochemical workstation (Shanghai Chenhua Co., China) under a phosphate buffer (0.1 M, pH 7.0). A conventional three-electrode system was adopted, with Nafion/GOD/CILE as the working electrode, a Pt wire as the counter and a saturated calomel electrode (SCE) as the reference. Prior to the experiment, the buffer solution was purged thoroughly with high purity nitrogen for at least 20 min, and a nitrogen atmosphere was then kept over the solution in the cell. Cyclic voltammetry (CV) experiments were performed between −0.1 and −0.8 V with a scan rate of 0.1 V/s. Chronoamperometric measurements were performed by switching the potential from 0 V (precondition for 20 s) to −0.5 V. The surface morphologies of the prepared electrodes were characterized on an S-4700 scanning electron microanalyzer (Hitachi, Japan) with an acceleration voltage of 15 kV. Fresh human serum samples were analyzed by a glucose oxidase method on an AU5421 chemistry analyzer (Olympus, Japan) in a local hospital. All experiments were performed at room temperature.

Electrode preparation

Mixtures of paraffin and [EIm][TfO] in various volume ratio (*v*/*v*) were ultrasonically dispersed for 5 min, then 15 μl of the mixture (as a binder) and 50 mg of graphite powder were carefully mixed in an agate mortar. A portion of homogeneous paste was packed firmly into a glass tube (3-mm diameter) and the electrical contact was established via a copper steel handle. The resulting electrode was recorded as CILE. A fresh surface was obtained by polishing the electrode on a piece of weighing paper and rinsed with double-distilled water just before use. GOD/CILE was prepared by simply dropping 5 μl 15 mg/mL GOD on the surface of CILE and dried at 4 °C for 6 h.

Finally, 3 μl of 2% Nafion solution was cast on the electrode surface to prevent the loss of the enzyme molecules and to improve the anti-interferent ability of the biosensor. The Nafion/GOD/CILE was stored at 4 °C when not in use.

Results and discussion

Optimization of the PIL–paraffin composite ratio

The attractive behavior of the protic ionic liquid ([EIm][TfO]) would be advantageous to fabricate PIL-carbon paste biosensors. However, the high background current limits its largely use as a binder, so it is important to find an optimal concentration of [EIm][TfO] [32]. In the process of making the CILE, the amount of graphite was 50 mg and the usage of binder was 15 μl; [EIm][TfO] and paraffin were mixed with different ratios. Figure 1a shows the effect of the PIL content upon CVs in a 5-mM potassium ferricyanide solution. With increasing the ratio from 0/15 to 4/11, the current peaks increased as well, and the reversibility was greatly improved. However, a dramatic increase in background current was observed when the ratio exceeds 2/13. On Nafion/GOD/CILEs made with these CILE, similar behaviors were discovered (0.1 M PBS buffer, pH=7.0) (Fig. 1b). The cyclic voltammograms of GOD were also affected by the different loading of ionic liquid. From the results in Fig. 1a, b, the ratio of 2/13 was chosen for the subsequent work.

Morphologies of CILE and GOD/CILE

Since [EIm][TfO] is a hydrophilic compound with high viscosity, it could fill into the layer of graphite powder as a bridge to the isolated carbon flake. The SEM image (Fig. 2a) of CILE showed a uniform and smooth surface [33, 34]. After CILE was further coated with GOD molecules, the aggregation of the immobilized GOD molecules was highly dispersed and showed a uniform snowflake structure (Fig. 2b). So the PIL was favorable to the immobilization of enzyme molecules.

Direct electron transfer of GOD on the CILE

The cyclic voltammograms of Nafion/GOD/CILE in N_2-saturated 0.1 M PBS (pH=7.0) were shown in Fig. 3. A pair of well-defined and nearly symmetric redox peaks could be observed as for the direct electron transfer of GOD (Fig. 3c). The anodic peak potential (E_{pa}) and cathodic peak potential (E_{pc}) were located at −0.437 and −0.515 V, respectively, at scan rate of 100 mV s^{-1}. The formal potential ($E^{0'}$=−0.476 V) is near the standard electrode potential of −0.46 V (vs. SCE) for FAD/$FADH_2$ at pH 7.0 (25 °C) [14], suggesting that most GOD molecules retain their native structure after immobilization on the CILE [13]. A 1:1 ratio of cathodic to anodic current intensity indicates that the electrochemical reaction is almost reversible. However, under the same conditions, but in the absence of GOD, bare CILE does not show any perceivable response (Fig. 3a, dot curve). When only GOD was immobilized on the CPE (carbon paste electrode) in the absence of the PIL, the cyclic voltammogram showed a small response of GOD (Fig. 3b, dash curve), which was much smaller than that of Nafion/GOD/CILE. Thus, the PIL [EIm][TfO] played an important role in facilitating the direct electron transfer between GOD and CILE.

The cyclic voltammograms of Nafion/GOD/CILE at various scan rates were investigated (Fig. 4a). With the increasing scan rate, the anodic peak potential and the cathodic peak potential of GOD was barely shifted. The peak currents (I_p) are proportional to the scan rate (ν) in the range from 40 to 260 mV/s (linear regression equations: I_{pc} (microamperes)=4.911+0.0522ν (millivolts per second), R=0.998; I_{pa}(μA)=−1.295–0.0408ν (millivolts per second), R=0.999), as shown in Fig. 4b, which indicates a surface-controlled electrode process for GOD redox chemistry. The electron transfer rate constant (k_s) can be calculated according to the model of Laviron [35]. Taking a charge transfer coefficient α of 0.56 and a scan rate of 100 mV/s,

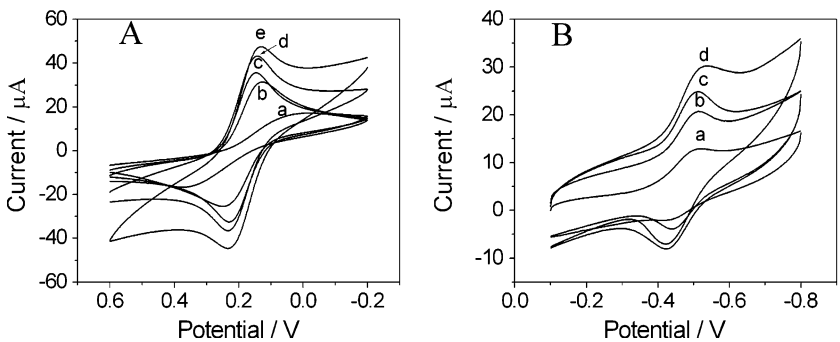

Fig. 1 The CVs of **a** CILE in 5 mM potassium ferricyanide and **b** Nafion/GOD/CILE in N_2-saturated PBS (0.1 M, pH 7.0) with different ratios of PIL and paraffin: 0/15 (*a*), 1/14 (*b*), 2/13 (*c*), 3/12 (*d*), 4/11 (*e*). Scan rate is 100 mV/s

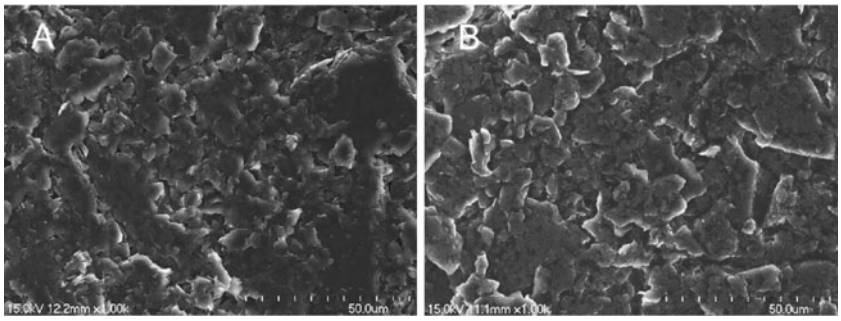

Fig. 2 SEM images of CILE (**a**) and GOD/CILE (**b**)

ΔE_p=78 mV, and then the rate constant k_s was ca. 3.98 s^{-1}. It is much larger than that of 1.30s^{-1} for Nafion/GOD–GNPs/GC electrode [13], 3.0 s^{-1} for GOD/SWCNT-CHI/GC [12] and 2.12 s^{-1} for GOD-IL-GNP-ILSWNT/GCE [8]. According to the equation $I_p=n^2F^2\nu AI^*/4RT=nFQ\nu/4RT$ [36], from the integration of reduction peaks of Nafion/GOD/CILE at scan rates less than 260 mV/s, the average surface coverage (I^*) of GOD is calculated to be 3.077×10^{-10} mol cm^{-2} from the slope of the I_p–ν curve. This value is even larger than the value reported (1.27×10^{-10} mol cm^{-2}) for GOD-IL-GNP-ILSWNT/GCE [8]. These results suggested that PIL [EIm][TfO] could improve the electron transfer rate and provide a large area for enzyme immobilization.

Cyclic voltammograms of Nafion/GOD/CILE show a strong dependence on solution pH, as shown in Fig. 5a. Obviously, with increasing pH from 4.92 to 8.00, the peak potentials shifted towards the negative direction; the peak currents increase to a maximum at around pH=7 and then decrease. $E^{0\prime}$ has a linear relationship with pH (the linear regression equation is $E^{0\prime}$=−0.174–0.0456 pH, R=0.998) (Fig. 5b), the slope is nearly close to the theoretical value (−58.6 mV/pH) [13]. It proves that two protons and two electrons participate in the electrochemical reaction of the GOD immobilized on CILE. The redox reaction process between GOD and electrode can be summarized as following:

$$\mathrm{GOD-FAD}+2e^-+2H^+ \longleftrightarrow \mathrm{GOD-FADH_2} \qquad (1)$$

Enzymatic activity of immobilized GOD

Cyclic voltammetric experiments demonstrated that the immobilized GOD still retained its electrocatalytic activity, as show in Fig. 6. In the oxygen-free 0.1 M PBS, a couple of well-defined redox peaks could be observed (Fig. 6, curve a), which could be attributed to the direct electron transfer between the GOD and electrode. When PBS was saturated with O_2, a significantly increased reduction peak current was observed together with the decrease of oxidation peak current (Fig. 6, curve b), which demonstrates that Nafion/GOD/CILE catalyzes the oxygen reduction (see Eqs. 1 and 2). Chronoamperometry was employed to further characterize the electrocatalytic activity of the immobilized GOD. The plot of chronoamperometric measurements is inseted in Fig. 6. Due to the biosensor that catalyzes the oxygen reduction, the current density is greatly enhanced in the oxygen-saturated PBS (insert curve b), which is in good

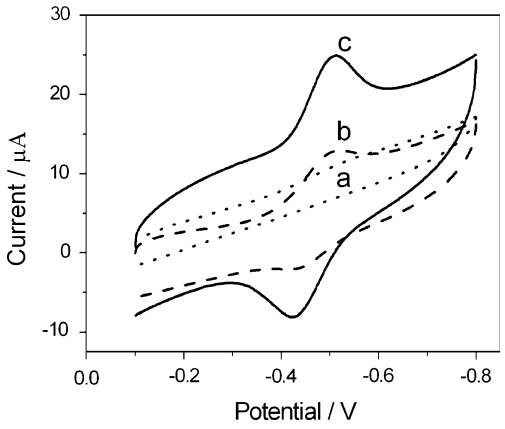

Fig. 3 The CVs of **a** bare CILE (*dotted line*), **b** Nafion/GOD/CPE (*dashed line*), and **c** Nafion/GOD/CILE (*solid line*) in N_2-saturated 0.1 M PBS (pH 7.0), scan rate at 100 mV/s

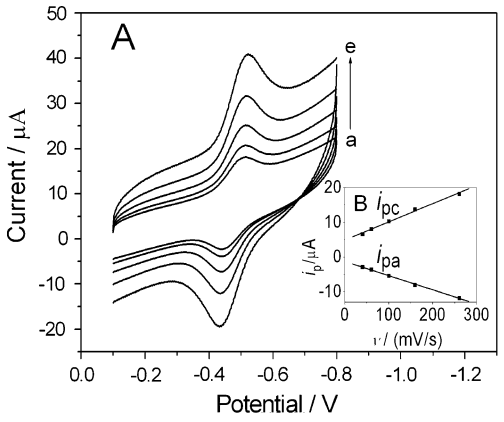

Fig. 4 **a** The CVs of Nafion/GOD/CILE in N_2-saturated 0.1 M PBS (pH 7.0) at various scan rates. The scan rate is 40, 60, 100, 160, and 260 mV/s (from *a* to *e*). *Inset* plot **b** relationship between scan rate and the cathodic and anodic peak current

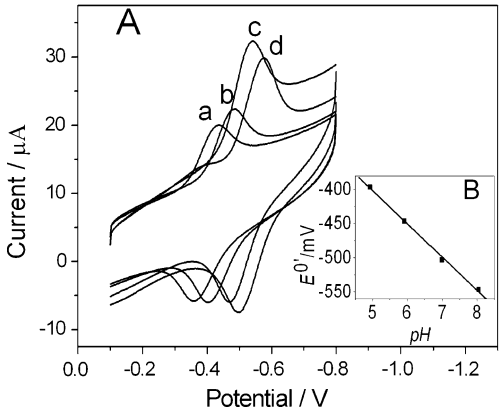

Fig. 5 **a** The CVs of Nafion/GOD/CILE in 1/15 M PBS at pH 4.92 (*a*), 5.91 (*b*), 6.98 (*c*), and 8.00 (*d*), scan rate is 100 mV/s. *Inset* graph **b** is the plot of the formal potential $E^{0\prime}$ vs. pH

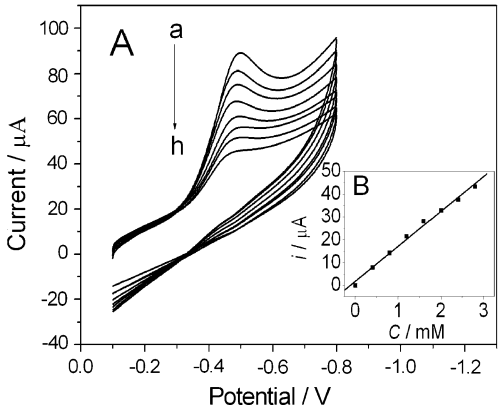

Fig. 7 **a** The CVs of Nafion/GOD/CILE in oxygen-saturated 0.1 M PBS (pH 7.0) with different glucose concentrations *C/mM* (from *a* to *h*): 0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8. The scan rate is 100 mV/s. *Inset* plot **b** is the relationship between electrocatalytic current decrease ΔI and glucose concentration

agreement with the CV measurements. The results indicated that GOD in the film retained its bioactivity [37].

$$\mathrm{GOD-FADH_2 + O_2 \rightarrow GOD-FAD + H_2O_2} \quad (2)$$

Adding glucose to the oxygen-saturated 0.1 M PBS, the decrease of the reduction current was obtained (Fig. 7a). The higher glucose concentration caused more of the reduction current to decrease. The biocatalytical process for the oxidation of glucose in the presence of GOD can be summarized as in Eq. 3:

$$\mathrm{GOD-FAD + glucose + O_2} \rightarrow \mathrm{GOD-FADH_2 + gluconolactone} \quad (3)$$

The enzyme-catalyzed reaction that occurred resulted in the decrease of the oxidized form of GOD on the electrode surface. So the reduction currents of GOD were decreased. The decrease of reduction current is linear against the concentrations of glucose ranging from 0.005 to 2.8 mM. The calibration curve corresponds to the equation of calibration I_{pc} (microamperes)=2.255+14.96C (millimolar) with a correlation coefficient (R) of 0.996 (Fig. 7b). The sensitivity was found to be 14.96 μA mM^{-1} (with an electrode area of 7.06 mm^2). This value is much greater than that of 1.77 μA mM^{-1} for an aprotic IL modified GOD biosensor [20]. The detection limit was estimated at 2.5 μM with a signal-to-noise ratio of 3. The apparent Michaelis–Menten constant (K_m) is an important parameter to reveal enzyme–substrate reaction kinetics, K_m for this enzyme-based electrode was estimated to be 1.53 mM, which was much smaller than previously reported [38–41], indicating that the immobilized GOD possesses higher enzymatic activity and the Nafion/GOD/CILE exhibits a higher affinity toward glucose. The suitable microenvironment due to effect of PIL [EIm][TfO] might contribute to the improvement of the affinity and good performances of the biosensor. For further comparison, the analytical performance and some electrochemical constants of the proposed sensor and some other glucose biosensors based on the direct electron transfer of GOD were listed in Table 1.

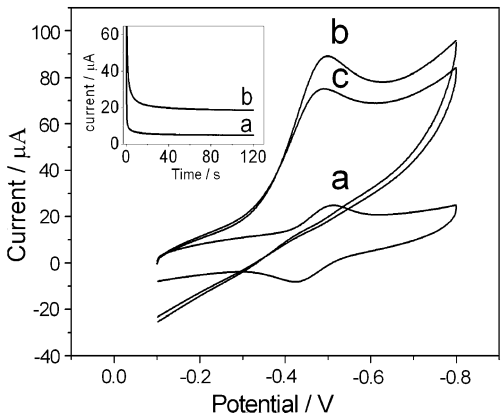

Fig. 6 The CVs and chronoamperometrics (CAs) (*inset*) of Nafion/GOD/CILE in 0.1 M pH 7.0 PBS, with **a** oxygen-free solution, **b** oxygen-saturated solution, **c** after addition of 0.8 mM glucose in **b** solution. Scan rate: 100 mV s^{-1}

Stability and reproducibility of Nafion/GOD/CILE

The long-term stability of Nafion/GOD/CILE was evaluated by examing the cyclic voltammetric peak currents of GOD on CILE. After continuously scanning for 300 cycles, there was only a 5.3% decrease of the anodic peak current, indicating the immobilized GOD has good stability. The storage stability of Nafion/GOD/CILE was also estimated. After the electrode was kept at 4 °C in 0.1 M PBS (pH 7.0)

Table 1 Comparison of different modified biosensors based on the direct electron transfer of GOD

Electrode	k_s	Sensitivity	Detection limit	K_m	References
GOx/PPMH/GC	1.32 cms^{-1}		0.05 mM	0.64 mM	[6]
GOx/$NdPO_4$ NPs/CHIT/GC	5.0 s^{-1}	1.92 μA mM^{-1}	0.08 mM	2.5 mM	[9]
Nafion/GOD–GNPs/GC	1.3 s^{-1}	6.5 μA mM^{-1} cm^{-2}	3.4×10^{-2} mM	4.6 mM	[13]
GOx-mesoFe/C-Nafion/Pt	0.49 s^{-1}	27 μA mM^{-1} cm^{-2}	0.08 mM	6.6 mM	[15]
GOD/CdS/PGE		7.0 μA mM^{-1}	0.05 mM	5.1 mM	[38]
CS-GOD-CdS/ACNTs-Pt_{nano}	3.8 s^{-1}	4.5 μA M^{-1}	4.68×10^{-2} mM	11.86 mM	[41]
Nafion/GOD/CILE	3.98 s^{-1}	14.96 μA mM^{-1}	2.5×10^{-3} mM	1.53 mM	This paper

k_s electron transfer rate constant, K_m apparent Michaelis–Menten constant, *DHP* dihexadecylphosphate, *GE* graphite electrode, *mesoFe/C* magnetic mesoporous carbon material, *GNPs* gold nanoparticles, *CS*, *CHIT* chitosan, *PPMH* 1,10-phenanthroline monohydrate, *ACNTs* aligned carbon nanotubes, *PGE* pyrolytic graphite electrode

for 30 days, 95% of its initial current response for glucose was retained. The reproducibility of Nafion/GOD/CILE was examined at a glucose concentration of 0.8 mM. The RSD (relative standard deviation) is 2.5% for six successive measurements. The presence of PIL is very effective for the retention of the enzyme activity. The effects of interference are also tested in the presence of different concentrations of uric acid and ascorbic acid, no significant change of reduction peak current can be observed, which shows the good selectivity of enzyme electrode.

Preliminary application of the Nafion/GOD/CILE

To illustrate feasibility of the Nafion/GOD/CILE in practical analysis, fresh human serum samples were first analyzed by the glucose oxidase method in a local hospital. As a standard method for clinical glucose sample detection, the operating procedures are as follows: glucose present in the serum is oxidized by the glucose oxidase to gluconic acid with the liberation of hydrogen peroxide, which is converted to water and oxygen by the enzyme peroxidase (POD). An oxygen acceptor, 4-aminophenazone, takes up the oxygen and together with phenol forms a pink colored chromogen which can be measured at 515 nm. The same serum samples were tested with the developed biosensor. There was no pretreatment other than the dilution of the samples (500-fold dilution). The detection results are in good agreement with each other, as shown in Table 2. The recoveries were determined with the standard addition method in serum samples with the biosensor. The average recoveries were ranged between 95% and 105% for three determinations. Compared to the clinical detection method, the present method based on the biosensor has many advantages such as simple, convenient, lower cost, and reliability.

Conclusions

In summary, a protic ionic liquid, [EIm][TfO], was used to make an ionic liquid modified carbon paste electrode (CILE). GOD was then successfully immobilized on the surface of the electrode. The electrochemical behavior of GOD at the PIL modified electrode was carefully investigated in 0.1 M PBS (pH 7.0). Compared with its response at CPE, the direct electrochemistry of GOD at the PIL-based electrode was improved dramatically. The further experimental results confirmed that the immobilized GOD exhibited a high electrocatalytic activity towards glucose. The PIL matrix provided a unique microenvironment around the enzyme, in which a high enzyme activity was retained, resulting in high sensitivity and excellent stability of the enzyme. The good properties of the modified electrode implied that PILs could be applied to provide a promising strategy for the development of biosensors.

Table 2 Determination of glucose in human serum samples

Sample no.	By hospital (mM)	This method (mM)	RSD (%)	Added (mM)	Found (mM)	Recovery (%)
1	10.7	11.3	3.91	0.40	0.39	97.5
2	6.7	6.5	6.57	0.40	0.41	102.5
3	7.3	7.5	2.43	0.40	0.42	105.0
4	5.5	5.9	5.59	0.40	0.38	95.0

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