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Electrochemical study of mono-6-thio-β-cyclodextrin/ferrocene capped on gold nanoparticles: Characterization and application to the design of glucose amperometric biosensor

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ABSTRACT

A novel amperometric glucose sensor based on inclusion complex of mono-6-thio-β-cyclodextrin/ferrocene capped on gold nanoparticles (GNPs/CD–Fc) and glucose oxidase (GOD) was described. The inclusion complex of mono-6-thio-β-cyclodextrin/ferrocene capped on gold nanoparticles played an effective role of an electron shuttle and allowed the detection of glucose at 0.25 V (versus SCE), with dramatically reduced interference from easily oxidizable constituents. The sensor (GNPs/CD–Fc/GOD) showed a relatively fast response time (5 s), low detection limit (15 μM, S/N = 3), and high sensitivity (ca. 18.2 mA M^{-1} cm^{-2}) with a linear range of 0.08–11.5 mM of glucose. The excellent sensitivity was possibly attributed to the presence of the GNPs/CD–Fc film that can provide a convenient electron tunneling between the protein and the electrode. In addition, the biosensor demonstrated high anti-interference ability, stability and natural life. The good stability and natural life can be attributed to the following two aspects: on the one hand, the fabrication process was mild and no damage was made on the enzyme molecule, on the other hand, the GNPs possessed good biocompatibility that could retain the bioactivity of the enzyme molecules immobilized on the electrode.

1. Introduction

Amperometric enzyme electrodes possess the advantages of combining the specificity of the enzyme for recognizing particular target molecules with the direct transduction of the rate of reaction into the current [1]. Enzyme electrodes, particularly the glucose electrode, have been subject to numerous studies devoted to their optimization for both biomedical and bioprocess control applications [2]. For glucose sensors, this enzyme is well-studied, inexpensive, stable and particularly applied in clinical and chemical analysis [3]. Nevertheless, there is need to improve the sensitivity and stability of glucose sensors due to the great demand for blood glucose measurements in control and treatment of diabetes. Recently, Adam Heller and Ben Feldman have given a wonderful review about electrochemical glucose sensors and their applications in diabetes management [4].

The oxidase-based amperometric biosensors previously relied on the immobilization of oxidase enzymes on the surface of various electrodes. However, electron transfer efficiency of redox enzymes is poor in the absence of mediator, because enzyme active sites are deeply embedded inside the protein. The sensitivity of resulted biosensors can be significantly improved by the immobilization of mediators in the matrices [5–9]. Among the different mediators described in the literature, ferrocene (Fc) and its derivatives, first reported by Cass et al. [5], have proved to be the most efficient electron transfers for the GOx enzymatic reaction. There are a lot of cases about ferrocene (Fc) and its derivatives introduced to enzyme biosensor as the mediator [10–16]. However, leakage has been a main problem for the entrapment of mediators due to their low molecular weight in polymer matrices [8]. In order to prevent the leakage of mediator, mediator can be linked covalently with polymer or with high molecular weight compounds before immobilization on the surface of electrode [8,17]. Gorton et al. [18] studied ferrocene-containing siloxane polymer modified electrode surface with a poly (ester-sulfuric acid) cation-exchanger to improve the stability of the mediator. Another alternative method is to synthesize a few Fc derivatives with specific functional groups [19,20], but the preparation methods are complicated. For instance, Jönsson et al. [19] used hydroxymethyl Fc and anthracene carboxylic acid to synthesize anthracene substituted ferrocene.

The other alternative method to increase the stability of Fc and its derivatives is the formation of inclusion complex with cyclodextrin (CD), a class of torpidly shaped cycloamyloses with a hydrophilic outer surface and a hydrophobic inner cavity, which makes the dissolubility of Fc decrease. Several investigations have been made to study the characterization of interacting Fc–CD

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system and their roles [21–23]. Liu et al. [21] developed the sensitive biosensor for glucose by immobilizing glucose oxidase in β-cyclodextrin via cross-linking and by including ferrocene in the cavities of dextrin polymer via host–guest reaction. Zhang et al. [22] successfully used ferrocene with β-cyclodextrin to prepare β-CD/Fc inclusion complex modified carbon paste electrode. The water-soluble inclusion complex of 1,1-dimethylferrocene with (2-hydroxypropyl)-β-CD has been used in bioelectrocatalysis [23].

Recently, nanomaterials, such as gold (Au) [24–27] and magnetic nanoparticles have attracted much interest in the construction of biosensors due to their unique chemical and physical properties. Gold nanoparticles, in particular, have been widely used to construct biosensors because of their excellent ability to immobilize biomolecules and at the same time retain the biocatalytic activities of those biomolecules. In this paper, gold nanoparticles were capped by inclusion complex between mono-6-thio-β-cyclodextrin and ferrocene through –SH, which resulted into stable fixation of ferrocene on the surface of gold nanoparticles. Then, the glucose biosensors were constructed by using GNPs/CD–Fc as the building block. The composite nanoparticles showed excellent efficiency of electron transfer between the GOD and the electrode for the electrocatalysis of glucose.

2. Experimental

2.1. Reagents

Glucose oxidase (GOD) (EC1.1.3.4, Type VII-S, 108 U/mg) from *Aspergillus niger* was purchased from Amresco. Ferrocene was obtained from the Shanghai Chemical Reagent Pharmaceutical Factory. Hydrogen tetrachloroaurate tetrahydrate ($HAuCl_4{\cdot}4H_2O$) and sodium borohydride ($NaBH_4$) were purchased from Shanghai Chemical Reagents Company. Mono-6-thio-β-cyclodextrin was synthesized according to the literature [28]. Other reagents used in this paper were of analytical grade and used as commercially available. Double distilled and sterilized water was used to prepare all solution.

2.2. Apparatus

The film of GNPs/CD–Fc/GOD was characterized by scanning electron microscope (SEM). SEM was taken by Philips XL-30ESEM operating at an accelerating voltage of 20 kV. The morphology and size of GNPs/CD–Fc after adding glucose oxidase aqueous solution were investigated on a Philips TECNAI-12 transmission electron microscope (TEM) instrument operated at an accelerating voltage of 120 kV. Images of GOD in GNPs/CD–Fc organized assemblies were observed by TEM after negative staining with sodium phosphotungstate. All electrochemical experiments were carried out with a CHI660c electrochemical workstation (Chenghua, Shanghai) with a three-electrode cell. A platinum disk electrode (diameter: 1 mm) was used as a working electrode. The reference electrode was a saturated calomel electrode (SCE). All potentials reported in this paper were against SCE. A platinum wire was used as the counter electrode. All measurements were carried out in a thermostated cell at 25 °C. The aqueous electrolyte solutions were prepared in 0.1 M PBS buffers (Na_2HPO_4–NaH_2PO_4) in which the pH was measured as 7.0 using a PHs-25 pH-meter (Leizi Instrumental Factory, Shanghai). Electrolytic solutions were purged with highly purified nitrogen for at least 20 min prior to the series of experiments.

2.3. Construction of the GNPs/CD–Fc/GOD modified electrode

Firstly, the inclusion complex was synthesized. Mono-6-deoxy-6-(*p*-tolylsulfonyl)-β-cyclodextrin (β-CD–SH) (0.5 g) and Fc (1.6 g) were dissolved by 20 mL DMF in a 100 mL conical flask. Then, the solution should be stirred continually at least 24 h at room temperature. Finally, 20 mL ethanol was added into the solution. Immediately, a great deal of yellow crystal precipitate was occurred, which was the inclusion complex of Fc with β-CD–SH. The solubility of inclusion complex was weak in ethanol. The inclusion complex was obtained by filtrating and washed with ethanol and water for three times in turn, respectively, to clean the residual guest and host monomers. Then it was dried in a vacuum oven at 50 °C for 24 h.

Secondly, CD–Fc capped gold nanoparticles were prepared by the reduction of $AuCl_4^-$ with a similar method described before [28]. $NaBH_4$ and CD–Fc were dissolved in 10 mL of DMF, and $HAuCl_4$ in 10 mL of DMF was quickly added into the mixture of $NaBH_4$ and CD–Fc. The molar ratio of [CD–Fc]/[$HAuCl_4$] was 1:1. After 24 h stirring at room temperature, the precipitate was collected by centrifugation and washed with 25 mL of DMF for three times and then with 25 mL ethanol/water (9:1 in volume) for four times. At last, the precipitate, GNPs/CD–Fc, was dried at 60 °C in a vacuum oven for 24 h and characterized by TEM.

Finally, the working electrode (diameter: 1 mm) was polished to a mirror with 0.05 μm alumina aqueous slurry and rinsed with water in an ultrasonic cleaning bath before use. GOD was also dissolved in deionized and decarbonated water with a concentration of 5 mg mL^{-1}. The colloidal suspension of GNPs/CD–Fc (1 mg mL^{-1}) was prepared by dispersing GNPs/CD–Fc in deionized and decarbonated water. A defined amount of the aqueous mixture (GOD and GNPs/CD–Fc) was spread on the surface of the platinum disk electrode. The coating was dried in air at room temperature. Finally, the film was equilibrated under stirring for about 20 min in a phosphate buffer solution in order to allow hydrogel formation and remove unadsorbed enzyme. All electrodes were stored in PBS at 4 °C.

3. Results and discussion

3.1. Characterization of the GNPs/CD–Fc/GOD films

Fig. 1 shows SEM images of GNPs/CD–Fc film and GNPs/CD–Fc/GOD composite film. From Fig. 1A, GNPs/CD–Fc was found to form the three-dimensional island structure of film. However, when enzyme was absorbed onto GNPs/CD–Fc, the composite film was multi-layer and multi-pore structure which indicated that the composite film was highly porous in nature (Fig. 1B). At larger magnification in Fig. 1C, the distribution of the enzyme on the composite film can be clearly observed. The enzyme congregated and formed global congregation (diameter: 100–500 nm). The multi-layer and multi-pore structure of the GNPs/CD–Fc/GOD composite film would be of benefit to the fast electron shuttle among enzyme, mediator and substrate.

The morphology of GOD and GNPs/CD–Fc in aqueous solution may be obtained by TEM images. The image of GNPs/CD–Fc was shown in Fig. 2A. Because there were enough –SH to cap on the surface of the gold nanoparticles, stable gold nanoparticles with uniform distribution were obtained. However, when GOD was added into the solution of GNPs/CD–Fc, the gold nanoparticles were congregated and formed abnormal structure shown as in Fig. 2B. Because the morphology of biomacromolecules cannot be observed directly by TEM, specimen must be negative staining with heavy metal salt to increase contrast. Therefore, in order to observe the morphology of GOD, the specimen was negative staining with sodium phosphotungstate for 60 s. Fig. 2C showed that a mass of GOD were congregated together and a lot of gold nanoparticles were deeply embed into the congeries. The electron transfer between the redox center of GOD and electrode would be effectively promoted through the combination mode between GOD and GNPs/CD–Fc.

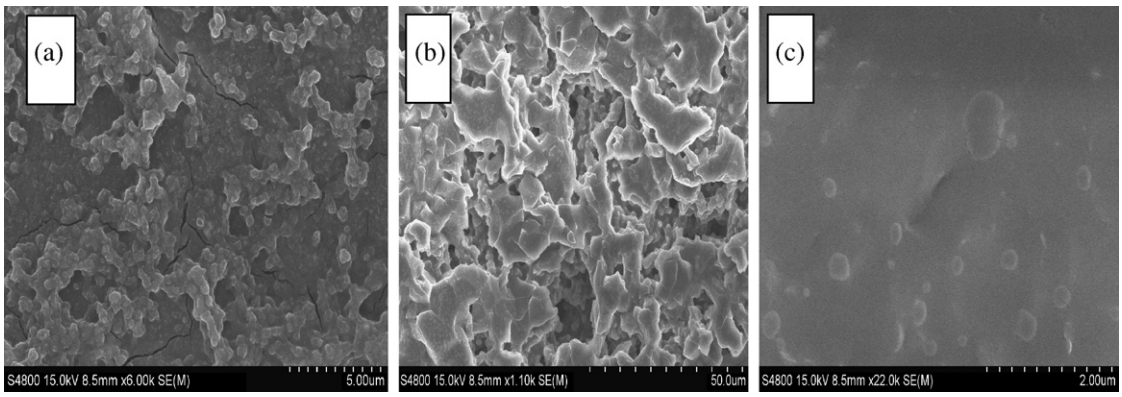

Fig. 1. SEM images of (A) GNPs/CD–Fc, and GNPs/CD–Fc/GOD with different magnifications of (B) 1100 and (C) 22,000.

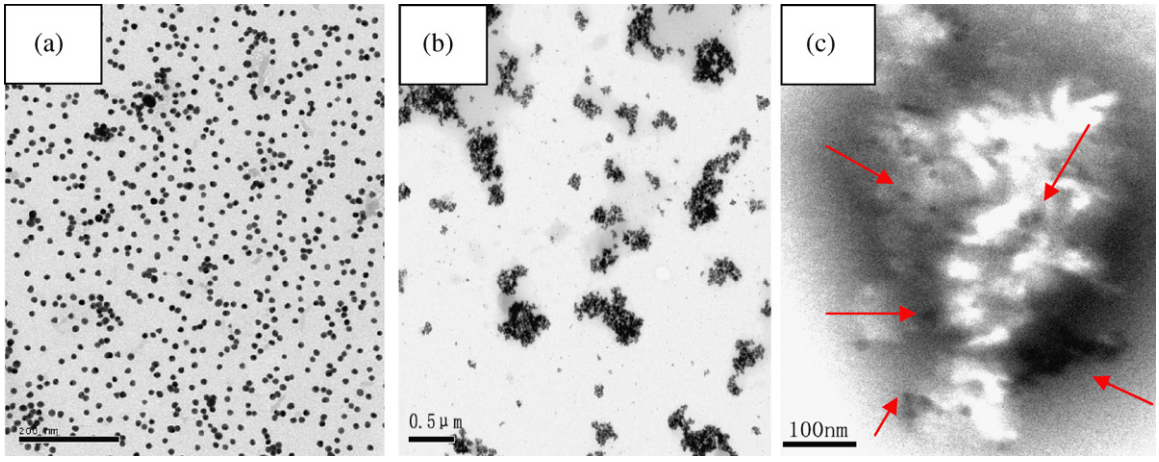

Fig. 2. TEM images of (A) GNPs/CD–Fc; (B) GNPs/CD–Fc/GOD without negative staining; (C) GNPs/CD–Fc/GOD negative staining with sodium phosphotungstate.

3.2. *Electrocatalytic oxidation of glucose at biosensor of GNPs/CD–Fc/GOD*

The GNPs/CD–Fc modified platinum electrode was cycled continuously in the potential range of −0.1 to 0.5 V (versus SCE) in 0.1 M PBS buffers. The current finally reached a steady state after 10 cycles. From Fig. 3A, there were well-defined anodic and cathodic peaks with the mean potential $\Delta E = (E_{pa} - E_{pc})$ at 75 mV (curve a in Fig. 3A). It corresponded to the reversible single electron oxidation of Fc to Fc^+, which indicated that the electrochemistry reaction was close to quasi-reversible state. Next, at the same condition, cyclic voltammogram of the enzyme electrode was carried out. In Fig. 3A, curve b showed well-defined anodic and cathodic peaks. Under the same conditions, neither glucose nor glucose oxidase showed any observable electrochemistry, so the peaks were still attributed to the redox reaction of Fc. Compared with the voltammogram of GNPs/CD–Fc modified platinum electrode, the anodic peaks shifted from 0.194 to 0.258 V and the ΔE increased 30 mV. When GOD was

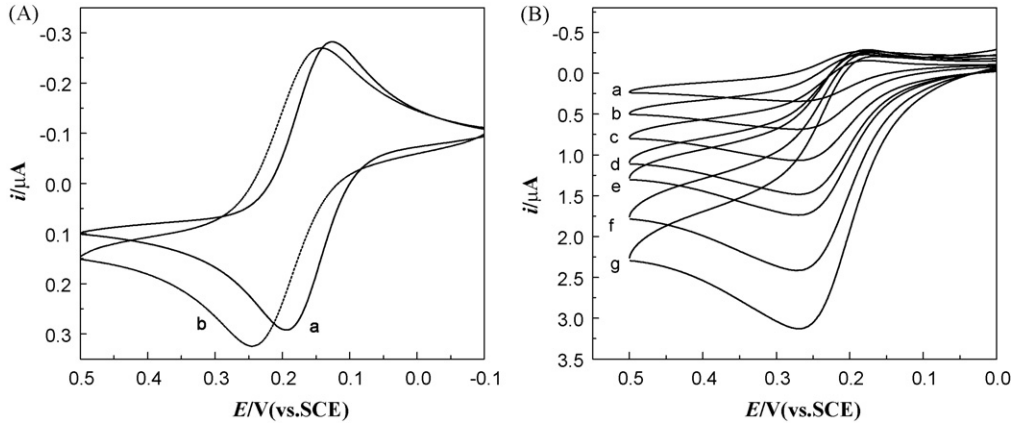

Fig. 3. (A) The cyclic voltammograms of (a) GNPs/CD–Fc (b) GNPs/CD–Fc/GOD modified electrode in 0.1 M PBS (pH 7.0) buffer solution. (B) The cyclic voltammograms on GNPs/CD–Fc/GOD modified electrode in 0.1 M PBS (pH 7.0) buffer solution with different concentration of glucose (mM): (a) 0; (b) 0.05; (c) 0.1; (d) 0.2; (e) 0.5; (f) 0.8; (g) 1.0. t = 25 °C, v: 10 mV s^{-1},

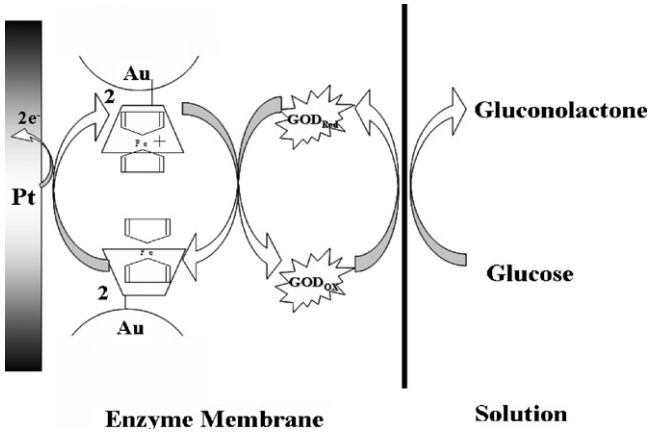

Fig. 4. Schematic illustration of sensing mechanism for electrocatalytic glucose on the GNPs/CD–Fc/GOD modified platinum electrode surface.

immobilized in the composite film, protein molecule blocked the charge transfer, which led to the positive shift of anodic peak and the value of ΔE increase. The phenomenon about the shifting of E_p upon immobilization of GOD was also observed in ferrocene-containing Nafion film [29]. At the same time, the current of anodic peak was larger than that of cathodic peak. The phenomenon was also observed in ferrocene-containing Nafion film. Upon the addition of glucose to the test solution, a very strong catalytic effect was observed: a dramatic increase in the oxidation current that was simultaneous with a gradual decrease of the reduction current (curves b–g in Fig. 3B). The decrease of the cathodic current was attributed to the reduction of oxidized form of Fc (Fc^+) by the reduced form of the enzyme (GODred), which was more efficient than the electro-reduction of the former at the base electrode. The oxidation wave was enhanced, since the reduced form of the mediator (Fc) was regenerated and then reoxidized at the base electrode. In this case, the mechanism of electron transfer from glucose to mediator was known to be of the "ping-pong" type which was shown in Fig. 4.

3.3. Optimization of experimental variables

In order to optimize the performance of the glucose biosensor based on GNPs/CD–Fc/GOD modified electrode, various experimental variables affecting the amperometric determination of glucose, such as the concentration ratio of GNPs/CD–Fc and GOD, GOD loading and pH of the solution, were investigated.

3.3.1. Influence of GOD loading

First, the effect of concentration ratio of GNPs/CD–Fc and GOD was studied. The response currents reached the maximum when the mass ratio of GOD and GNPs/CD–Fc was 1:5. The effect of GOD loading activity on the response characteristics of GNPs/CD–Fc/GOD modified electrode was examined in the presence of 1.0 mM glucose. The results showed that the current increased with the enzyme loading and the response currents reached the maximum when the GOD loading located at 12.7 $\mu g\,mm^{-2}$. However, when the GOD loading continuously increased, the current gradually decreased, which was due to the increasing resistance of the biomembrane. Furthermore, it is well known that enzyme denaturation often causes a considerable reduction in loss of biosensor response. Considering the response stability of the GNPs/CD–Fc/GOD biosensor, GOD loading for each test strip was set at 12.7 $\mu g\,mm^{-2}$ in this study.

3.3.2. Influence of pH

The value of pH is known to be one of the most important factors affecting the enzyme activity and stability, and hence the performance of enzyme electrodes. Lukachova et al. [29] also stated that pH values between 5.5 and 7 should be considered as being more appropriate for biosensors application, since most oxidases have pH optima in the neutral pH region. Thus, effects of pH on the current of the biosensor were determined in the range of pH 5–9 in the presence of glucose (1.0 mM). The results show that the highest response current can be obtained at pH 7. The pH value of the highest activity for GOD is 5.5 as reported by the supplier. In the case, the optimal pH value is larger than that for GOD highest activity (ca. pH 5.5), which is attributed to the presence of GNPs/CD–Fc, which may change the microenvironment of the enzyme and the surface charge distribution of the enzyme [30]. The experiment result also shows that the current sharply decreases at higher pH values. Wilson and Turner [31] ever reported that catalytic activity of GOD was rapidly lost below 2 or above 8 for pH. This result suggested that GNPs/CD–Fc addition may have no benefit to preventing GOD from alkaline inactivation. Moreover, the physiological pH value of blood is known to be near 7. As a consequence, pH 7 was selected for evaluating the performance of the glucose biosensor based on the GNPs/CD–Fc/GOD modified electrode.

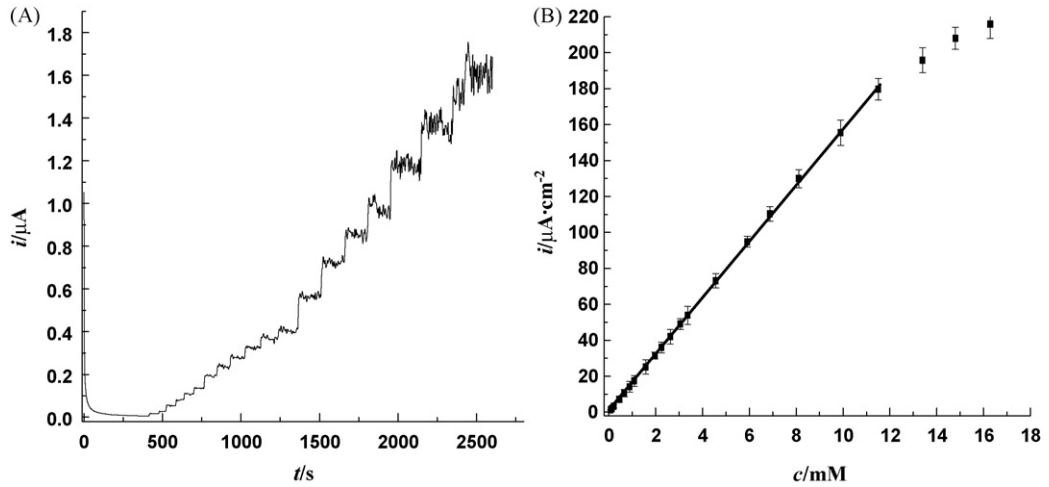

Fig. 5. (A) The i–t plot of GNPs/CD–Fc/GOD electrode response for glucose, under the optimum conditions; (B) Calibration curves of GNPs/CD–Fc/GOD for glucose, under the optimum conditions (0.1 M phosphate buffer solution with pH 7.0, at 25 °C, E_{app} = 0.25 V).

3.4. Performance of the chronoamperometric glucose biosensors

The electrocatalytic oxidation of glucose at GNPs/CD–Fc/GOD modified platinum electrode was also studied by amperometry. The potential of 0.25 V (versus SCE) was chosen as the applied potential for the amperometric determination of glucose. Fig. 5A showed the typical response curves for the GNPs/CD–Fc/GOD modified platinum electrode on successive injections of glucose to the stirring PBS at pH 7.0. Fig. 5B showed the corresponding linear range of the calibration curve obtained via the GNPs/CD–Fc/GOD modified platinum electrode. A clearly defined oxidation current proportional to the concentration of glucose was observed. The biosensor achieved 95% of the steady-state current within 5 s, with a linear range of 8×10^{-5} to 11.5×10^{-3} M. This kind of biosensor had a low detection limit of 1.5×10^{-5} M based on S/N = 3 and a high sensitivity of 18.2 mA M^{-1} cm^{-2}. The high sensitivity of the enzyme electrode can be attributed to the excellent adsorption ability, high electrocatalytic activity and good biocompatibility of the GNPs/CD–Fc composite nanoparticles. The apparent Michaelis–Menten constant (K_M) can be obtained by the analysis of the slope and the intercept of the plot of the reciprocals of the steady-state current versus glucose concentration. K_M value for GNPs/CD–Fc/GOD modified platinum electrode was 6.42 mM.

3.5. Effects of the interferences on the response of mediated enzyme biosensors

Generally, the influence of the presence of easily oxidizable endogenous and exogenous interferences was examined at their physiological normal levels on the biosensor response to glucose as reported in literature [32,33]. However, the normal concentration of these interferences (0.1 mM for ascorbic acid, 0.5 mM for uric acid, 0.2 mM for dopamine, 0.05 mM for *p*-acetaminophenol, and 0.02 mM for L-cysteine) was rather low compared with that of glucose (5.6 mM). The experiment results were shown in Table 1. Except for uric acid, the influences of ascorbic acid, dopamine, *p*-acetamidophenol and L-cysteine were quite weak. This was ascribed to the efficiency of the electrocatalytic process in the whole structure of the GNPs/CD–Fc composite film and also to the value of the low operating potential of 0.25 V versus SCE.

3.6. Reproducibility and stability of GNPs/CD–Fc/GOD biosensors

The reproducibility of biosensor was estimated by five GNPs/CD–Fc/GOD electrodes, which were prepared under the same condition and used to detect the current response of 1 mM glucose. The results revealed that the biosensors possessed satisfying reproducibility with a relative standard deviation (RSD) of 5.87% for 50 successive measurements (each sensor was performed 50 measurements). The storage stability of enzymatic biosensor in storage conditions (phosphate buffer of pH 7.0 at 4 °C) was investigated in the same phosphate buffer solution containing 1.0 mM glucose. In the storage stability test, 5 measurements of each biosensor were performed in each day during the period of the 30 days. The corresponding result showed that 82% response current was still retained after 30 days. The good stability can be attributed to the following aspects. Firstly, the fabrication process was mild and no damage was made on the enzyme molecule. Secondly, the GNPs possessed good biocompatibility that can retain the bioactivity of the enzyme molecules immobilized on the electrode. Finally, GOD can be immobilized efficiently on the film, which maintained a porous environment likely to impart fast mass transport issues into the composite film and thereby good sensitivity to the biosensor.

Table 1
Influence of interfering compounds on the response of GNPs/CD–Fc/GOD electrode.

Interfering compound	Physiological concentration (mM)	$(i_{G+I} - i_G)/i_G$ (%)
Ascorbic acid	0.1	3
Uric acid	0.5	18
3-Hydroxytyramine hydrochloride	0.2	5
p-Acetamidophenol	0.05	4
L-Cysteine	0.02	1.5

i_G: current response to 5.6 mM glucose; i_{G+I}: current response to 5.6 mM glucose in presence of interfering compound.

Table 2
Glucose content determination in serum sample.

Samples	Content determined by local hospital (mM)	Content determined by current method (average of five times) (mM)	Relative error (%)
1	4.17	4.35	−4.1
2	6.19	5.97	+3.7
3	4.88	4.99	−2.2
4	5.83	5.67	+2.8
5	4.26	4.43	−3.8

3.7. Real sample analysis

Human plasma samples were assayed to demonstrate the practical use of the GNPs/CD–Fc/GOD electrode. Fresh plasma samples were first analyzed in the local hospital with Olympus AU2700 biochemistry analyzer (Japan). The samples were then reassayed with the GNPs/CD–Fc/GOD electrode. A plasma sample (0.5 mL) was added into 5 mL PBS (pH 7.0), and the response was obtained at +0.25 V. The contents of glucose in blood can then be calculated from the calibration curve. The results, which were shown in Table 2, were satisfactory and agreed closely with those measured by the biochemical analyzer done by local hospital, indicating that the fabricated glucose biosensor had practical potential.

4. Conclusions

GOD can be effectively immobilized on GNPs/CD–Fc modified electrode. The immobilized GOD maintained its bioactivity and native structure. Fc was fitted on the surface of GNPs through inclusion complex with CD. This construction of the GNPs/CD–Fc/GOD modified electrode can decrease the leakage of mediator and improve the reproducibility and stability of GNPs/CD–Fc/GOD biosensors. From the cyclic voltammetry and amperometric measurements, the entrapped Fc was demonstrated to be redox active and function as electron shuttling agent between GOD and electrode. The use of artificial electron mediators to shuttle electrons from the redox center of the enzyme to the surface of the working electrode can effectively reduce the operating potential (0.25 V) and hence enhance selectivity and sensitivity.

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