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Amperometric sensor based on ferrocene-modified multiwalled carbon nanotube nanocomposites as electron mediator for the determination of glucose

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ABSTRACT

A kind of nanocomposite with good dispersion in water was prepared through covalent adsorption of ferrocenecarboxaldehyde on multiwalled carbon nanotubes (MWNTs) for electrical communication between glucose oxidase (GOD) and electrode. The ferrocene-modified multiwalled carbon nanotube nanocomposites (MWNTs-Fc) could be conveniently cast on electrode surfaces. With the aid of chitosan, GOD was then immobilized on the nanostructure film to form a reagentless amperometric sensor for glucose determination. FTIR spectra and cyclic voltammetry were used to characterize the nanocomposites. The presence of both ferrocene as mediator of electron transfer and MWNTs as conductor enhanced greatly the enzymatic response to the oxidation of glucose. The novel biosensor exhibited a fast response toward glucose with a detection limit of 3.0×10^{-6} mol/L and the linear range extended up to 3.8×10^{-3} mol/L.

Nowadays, amperometric enzyme-based biosensors constitute promising technology for various fields, such as chemical and biomedical analysis, pollution monitoring, biotechnology, and food and agricultural product processing [1–4]. Amperometric enzymatic biosensors have been considered to be the most suitable for biochemical analysis due to their good selectivity, sensitivity, rapid response, miniature size, and reproducible results [5]. 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 the resulting biosensors can be significantly improved by the addition of mediators in matrices.

Ferrocene and its derivatives are well-known substances as mediators [6–8] due to their various very desirable properties, e.g., relatively low molecular mass, reversibility, regeneration at low potential, and generation of stable redox forms [9]. Nevertheless, there are still several challenges concerning immobilization of electron-shuttling mediators on the electrode surface since low molecular weight soluble mediators can easily diffuse away from the electrode surface into the bulk solution when the biosensor is used continuously, which would lead to significant signal loss and greatly affect the performance and lifetime of the biosensor. This limitation can be dissolved through direct cross-linking of ferrocene derivatives with long poly(oxyethylene) chains [10] or conjugation with *m*-phenylenediamine polymer [11]. Another way is to bind covalently ferrocene derivatives to flexible dendritic or polymeric backbone and then the resulting materials are deposited onto the surface or incorporated into the carbon paste electrodes [12,13]. Ju and co-workers have reported a direct linking of ferrocene with bovine serum albumin, and the conjugate could be easily entrapped in sol–gel/ormosil films without leaching [14]. Sun and co-workers developed reagentless glucose biosensors based on ferrocene-branched chitosan matrix, which exhibited high sensitivity and a comparable linear range [15]. Our group proposed an amperometric biosensor based on ferrocene-modified silica [16] and Fe_3O_4@SiO_2 magnetic nanoparticles [17] as building blocks, which showed excellent efficiency of electron transfer for the electrocatalysis of glucose.

Carbon nanotubes (CNTs)[1] [18] have attracted much attention due to their high chemical stability, high surface area, unique electronic properties, and relatively high mechanical properties [19]. As electrode materials, this has led more recently to an upsurge of research on incorporating CNTs into enzyme-based biosensing platforms. Some of the electrode configurations employing CNTs that have been developed include the following: (a) physically adsorbed onto the nanotubes in solution then casting nanotubes onto electrodes [20,21], (b) microfabrication of nanoelectrode ensembles

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[1] *Abbreviations used:* FcCHO, ferrocenecarboxaldehyde; CNTs, carbon nanotubes; CS, chitosan; EDA, ethylenediamine; GOD, glucose oxidase; MWNTs-Fc, ferrocene-modified multiwalled carbon nanotube nanocomposites; MWNTs, multiwalled carbon nanotubes; $NaCNBH_3$, cyanoborohydride; PBS, phosphate-buffered saline; RSD, relative standard deviation; SWNTs, single-walled carbon nanotubes.

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and arrays either by adsorbing the enzyme or by first immobilizing its flavin adenine dinucleotide (FAD) center onto single-walled carbon nanotubes (SWNTs) and then reconstituting the enzyme [22,23], (c) entrapped the enzyme on CNTs by electropolymerization [24], or (d) assembled enzyme on multiwalled carbon nanotubes (MWNTs) wrapped by polyelectrolyte [16,25]. In fact, the high ratio with large surface area of CNT makes it possible to immobilize tethering molecules mediators with high density either via covalently bonding or via van der Waals interaction and show enhanced electrochemical detection capability [26–28]. Furthermore, the incorporation of carbon nanotubes into the poor electrical conductivity of the polymer matrices can improve the conductivity of the resulting matrices and facilitate the electron transfer among immobilized enzyme, mediator, and electrode [16,29,30].

In the present work, an amperometric biosensor for glucose was developed by entrapping glucose oxidase (GOD) in a new composite doped with MWNTs-Fc and chitosan (CS). Ferrocenecarboxaldehyde (FcCHO) was covalently bound onto the MWNTs with ethylenediamine (EDA) as cross-linker to achieve Fc-MWNTs conjugate. The prepared MWNTs-Fc/CS composite possessed high surface area, good mechanical stability, and good conductivity, which provided a compatible microenvironment for maintaining the activity of the immobilized enzyme and mediator, increased the mediator loading, and more importantly prevented the leakage of mediator. The designed MWNTs-Fc/CS membrane-modified electrode was successfully applied for the sensitive and selective assay of glucose.

Materials and methods

Chemicals and instruments

Glucose oxidase (from *Aspergillus niger* (EC 1.1.3.4), 150,000 units g^{-1}), ferrocenecarboxaldehyde, cyanoborohydride ($NaCNBH_3$), and chitosan (85% deacetylation) were purchased from Sigma-Aldrich and used as received. Multiwalled carbon nanotubes (with length of 5–15 μm, external diameter 10–20 nm, and surface area 40–300 m^2/g) were procured from Shenzhen Nanotech Port Co. (Shenzhen, China) and purified using literature techniques [31]. All other chemicals were of analytical grade and used without further purification. All the solutions were prepared using doubly distilled water.

FTIR spectra were recorded using a Nicolet 5700 FTIR. An Autolab PGSTAT30 (Eco Chemie) electrochemical system driven by GPES 4.9 software was used to collect electrochemical data. A conventional three-electrode cell assembly was used. The composite film-modified electrode was used as the working electrode. Ag/AgCl and platinum wire were used as reference and counter electrodes, respectively. The electrolyte solutions were purged with highly pure N_2 for at least 10 min to remove O_2 and kept under N_2 atmosphere during the measurements.

Functionalization of MWNTs

The functionalization of MWNTs was prepared according to Ref. [31]. In short, the purified MWNTs were suspended in anhydrous dimethyl formamide (DMF) in an ultrasonic bath. This dispersion (5 mL, 20 mg/mL) was immediately added to thionyl chloride ($SOCl_2$, 10 mL) and refluxed for 24 h to convert the carboxylic acids to acyl chlorides. The resulting nanotubes were rinsed over a 0.22-μm polycarbonate membrane filter with anhydrous tetrahydrofuran (THF) to remove excess $SOCl_2$. After being washed and ultrasoniced, they reacted with ethylenediamine (20 mL) for 3 days while the solution was stirred in order to form the intermediate at room temperature. Finally the carbon nanotubes were centrifuged and washed with water several times to remove the excess EDA and dried in a vacuum oven at 60 °C overnight. Then the aminated MWNTs (MWNTs-CO-$NH(CH_2)_2NH_2$) were obtained.

Preparation of MWNTs-Fc nanocomposite

The amount of 15 mg of the aminated MWNTs was dispersed in 5 mL doubly distilled water with ultrasonicating for 10 min, and then added to the FcCHO aqueous solution (10 mL, 7 mg/mL) with stirring at room temperature. After 1 h, $NaCNBH_3$ (200 mg) was added in the mixture, and left stirring for 24 h. Then the MWNTs-Fc nanocomposites were centrifuged, and washed with methanol and water several times to remove the dissociative FcCHO or any physically adsorbed FcCHO from the surface of MWNTs. Finally the MWNTs-Fc nanocomposites were redispersed in doubly distilled water with ultrasonicating.

Preparation of MWNTs-Fc/CS composite-modified electrode

The amount of 50 μL of 0.5 wt% CS solution [17] was mixed with 100 μL 3 mg/mL MWNTs-Fc with ultrasonic agitation over 15 min. Then, 5 μL of the resulting homogeneous solution was spread evenly onto the well-polished glassy carbon electrode (GCE) surface with a syringe and allowed to dry at 4 °C for 24 h to form a MWNTs-Fc/CS composite film. For the preparation of enzyme-incorporated composite film, a different amount of GOD was added to the mixture before casting. After the composite film-modified electrode was washed thoroughly with 0.1 M, pH 7.0, PBS, it was stored at 4 °C till use.

Results and discussion

Characteristics of MWNTs-Fc nanocomposite

MWNTs were first oxidized in a mixed solution of sulfuric and nitric acids by refluxing for 12 h. Then the freshly oxidized MWNTs were immediately added to thionyl chloride to convert the carboxylic acids of the MWNTs sidewalls to acyl chlorides, and to react with EDA to form MWNTs-CO-$NH(CH_2)_2NH_2$ [32]. The successful covalent attachment of EDA to MWNTs is proved by the FTIR measurement (Fig. 1a). IR absorption of the free amino group in EDA appeared at 1560 cm^{-1} due to the in-plane vibration mode of the N–H and C–N bonds. The bands at 2923 and 2852 cm^{-1} are due to the C–H stretch vibration. The aldehyde group of FcCHO can eas-

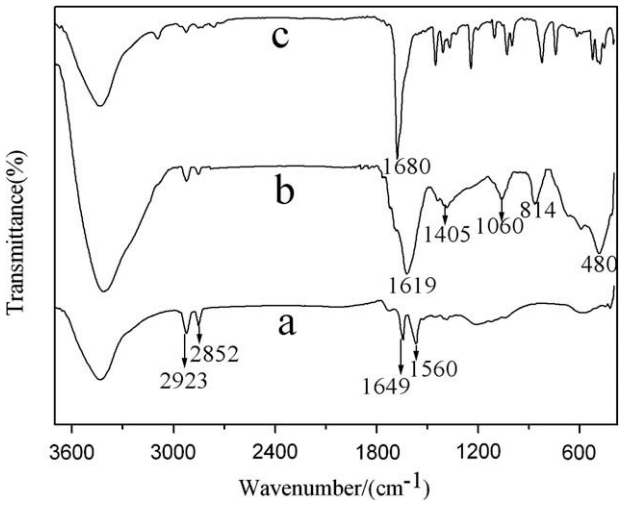

Fig. 1. FTIR spectra of MWNTs-EDA (a), MWNTs-Fc (b), and FcCHO (c).

ily react with the terminal amino groups on MWNTs-CO-$NH(CH_2)_2NH_2$ to form a high-function density of MWNTs-Fc composite through formation of a Schiff base and subsequent reduction by $NaCNBH_3$. The formation of MWNTs-Fc is confirmed by FTIR spectra (Fig. 1b). Compared to the MWNTs-EDA nanocomposite (Fig. 1a), besides the characteristic peak of alkane chains [2923 cm^{-1} $\nu_{as}(CH_2)$, 2852 cm^{-1} $\nu_s(CH_2)$], the ferrocene characteristic absorptions [1405, 1060, 814, and 480 cm^{-1}] of the MWNTs-Fc nanocomposite is clearly seen in Fig. 1b, which is in agreement with the previous report [33]. In addition, the free amino group at 1560 cm^{-1} for EDA (Fig. 1a) and the C=O group at 1680 cm^{-1} for FcCHO (Fig. 1c) were completely absent in parts, which meant that the aldehyde groups of FcCHO completely reacted with the amino groups of EDA to form Schiff bases and subsequent reduction by $NaCNBH_3$ to form the MWNTs-Fc nanocomposite.

Electrochemical behavior of the MWNTs-Fc/CS-modified electrode

The cyclic voltammogram of the MWNTs-Fc/CS-modified electrode exhibited a pair of well-defined redox peaks located at 0.33 and 0.28 V with the peak separation (ΔE_p) of 50 mV at 100 mV/s in PBS, which was attributed to the redox of Fc in the nanocomposite (curve a, Fig. 2A). The formal potential of 0.30 V, calculated from the average value of the cathodic and anodic peak potentials, shifted slightly compared with 0.36 V for a soluble redox couple, $FcCH_2NH_2$ [6]. Polymer microenvironment effects are likely to modify the free energy of the redox sites in this system. The electrochemical stability of the MWNTs-Fc/CS-modified electrode was checked by a repetitive potential sweep at a scan rate of 100 mV/s. The results showed that the peak currents maintained the same original intensity after 100 scans (curve b, Fig. 2A), which indicated that the electrochemistry of the MWNTs-Fc/CS-modified electrode is fairly stable and the leakage of Fc from the surface of the MWNTs-Fc/CS-modified electrode is neglectable. To further demonstrate the effect of MWNTs in the covalent immobilization of Fc, Fc/CS-modified electrode was prepared. With a continuous cyclic scanning, the voltammetric responses of the Fc/CS composite film-modified electrode decreased gradually (not shown), indicating the leakage of Fc from the electrode surface. It is evident that the stability of the modified electrodes prepared by this protocol is improved compared with the Fc/CS-modified electrode and the Fc-doped silica nanoparticle electrode whose peak current dropped by 30% after 100 repeated scans [34].

The effect of varying the scan rate on the performance of the electrode was also studied. With an increasing scan rate, the CV peak currents of the MWNTs-Fc/CS-modified electrode increased in the scan rate range of 10–800 mV/s, and ΔE_p was nearly independent of the scan rate (Fig. 2B), indicating that the Fc mediator was efficiently connected on GCE for facile charge transfer [35,36]. The cathodic and anodic peak currents increased linearly with the increase of the square root of scan rates (inset in Fig. 2B), suggesting that the electrochemical reaction of MWNTs-Fc/CS-modified electrode is a diffusion-controlled process [15,30].

Electrochemical response of GOD/MWNTs-Fc/CS-modified electrode to glucose

Since the modified electrode exhibits stable and reversible electrochemistry, it can be used as electron transfer mediator to shuttle electrons between analytes and the electrode. GOD was selected as a model enzyme to evaluate the electrocatalytic ability of the MWNTs-Fc/CS-modified electrode. Fig. 3 shows the CVs of the GOD/MWNTs-Fc/CS-modified electrode in the absence and presence of glucose in 0.1 M PBS. In the absence of glucose, the reversible electrochemical behavior of Fc was observed on the GOD/MWNTs-Fc/CS-modified electrode (Fig. 3a). After addition of 4.0 mM glucose, the anodic peak current increased dramatically and the cathodic current decreased (Fig. 4b), which clearly showed the catalytic properties of modified electrode to glucose. This reaction could be described by the well known following mechanism [34]: In the presence of glucose and enzyme GOD, glucose is oxidized to gluconolactone and the coenzyme $GOD_{(ox)}$ is converted to $GOD_{(red)}$. The resulting reduced form of the enzyme $GOD_{(red)}$ is then reoxidized by the ferrocene ion, yielding ferrocene, which in turn is reoxidized at the underlying electrode with generation of an amperometric catalytic anodic current.

The influence of the buffer pH is very essential to the sensitivity of the biosensors, because the pH affects not only the electrochemical behavior of Fc but also the bioactivity of GOD. The optimal pH reported for GOD is usually in the range of 6.5–7.5 [37–40], which varies with immobilization method and microenvironment around the enzyme. Thus the effect of pH was examined in the range of pH 5.5 to 8.5. This biosensor showed a maximum response at pH 7.0 (Fig. 4), which was consistent with other previous studies [30]. This optimum pH value is less acidic than the optimum pH value (5.5) reported for the free enzyme [41]. This is attributed to that the microenvironment of the enzyme has been changed by the immobilization and the resulting charge distribution of the enzyme surface has been changed [42]. As a consequence, the performance of the glucose biosensor was evaluated at pH 7.0 in this work.

Performance of the amperometric biosensor

The dependence of the enzyme electrode response on applied potential was investigated between 50 and 400 mV (not shown). The oxidation of ferrocene started at approximately 50 mV, and

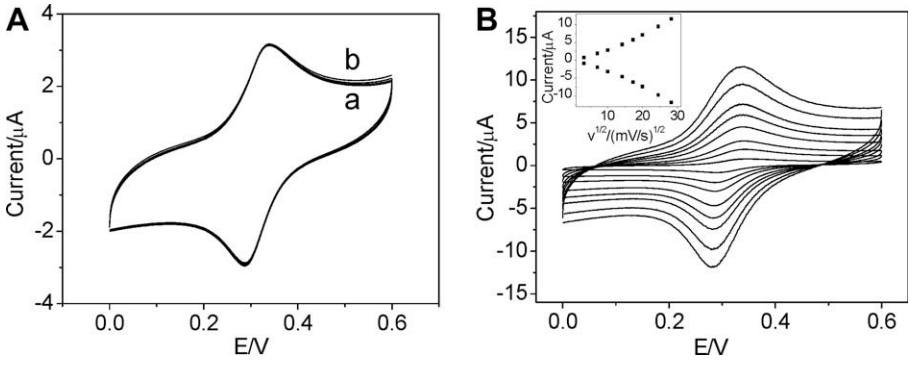

Fig. 2. (A) Cyclic voltammograms of MWNTs-Fc/CS nanocomposite-modified electrode at the first (a) and the 100th (b) scanning circles at 100 mV/s. (B) Cyclic voltammograms of MWNTs-Fc/CS nanocomposite-modified electrode in 0.1 M, pH 7.0, PBS at 10, 50, 100, 200, 300, 400, 600, and 800 mV/s (from internal to external). The inset is the plot of anodic and cathodic peak current vs $v^{1/2}$.

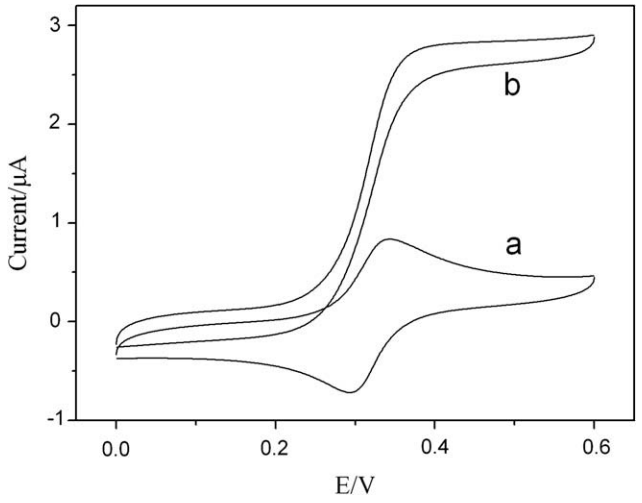

Fig. 3. Cyclic voltammograms of GOD/MWNTs-Fc/CS nanocomposite-modified electrode in the (a) absence and (b) presence of 3.0 mM glucose in pH 7.0 PBS at 10 mV/s.

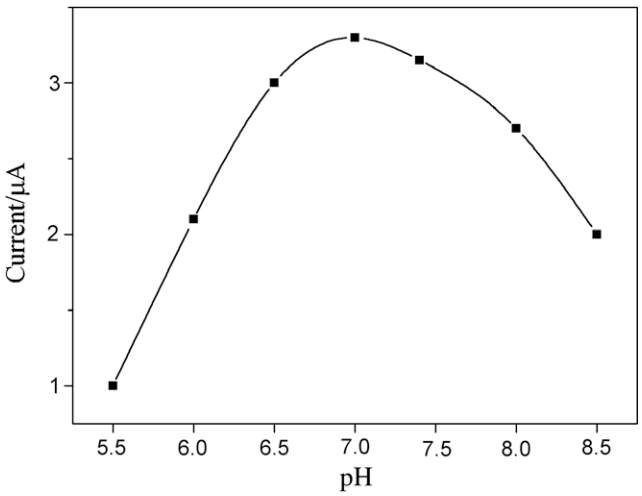

Fig. 4. Amperometric response of the GOD/MWNTs-Fc/CS film-modified electrode in N_2-saturated PBS (containing 4.0 mM glucose) of various pH. Potential: +350 mV.

reached a maximum response at 400 mV. However, several potential interferents (such as cysteine, uric acid, and ascorbic acid) would cause significant interference to the modified electrode response to glucose when higher potential was applied. Hence, the optimum applied potential of 350 mV was chosen throughout this study.

Fig. 5 shows the typical current–time responses at GOD/MWNTs-Fc/CS-modified electrode for successive addition of glucose. The time required to reach 95% of the maximum steady-state current was less than 5 s, indicating a fast response, which was mainly due to the existence of Fc as mediator of electron transfer and the well-conductive properties of the MWNTs [14]. With the increasing glucose concentration the amperometric response increased linearly in the range from 1.2×10^{-5} to 3.8×10^{-3} mol/L. The detection limit was 3.0×10^{-6} mol/L at a signal to noise ration of 3. The sensitivity was about 25 μA cm^{-2} mM^{-1}, which is much better than that of 0.28 μA cm^{-2} mM^{-1} in Ref. [43]. At high glucose concentrations a platform response is observed, showing a characteristic of the Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten constant (K_m^{app}), a reflection of the enzymatic affinity, is calculated to be 3.12 mM according to the Lineweaver–Burk equation (inset b in Fig. 5) [44]. This value is much smaller than the native GOD in solution (27 mM) [45], the biosensor prepared by sol–gel organic–inorganic hybrid material (20 mM) [46], and by covalent attachment of GOD to an Au electrode modified with gold nanoparticles (4.3 mM) [47]. The smaller K_m^{app} value means that the immobilized GOD possesses higher enzymatic activity and the proposed electrode exhibits a higher affinity to glucose. Compared with other glucose biosensors based on chitosan, the GOD/MWNTs-Fc/CS-modified electrode exhibited higher sensitivity and a comparable linear range [48]. In particular, the proposed method avoids using diffusional electron transfer mediators for the determination of glucose, which is necessary for traditional enzyme-based biosensors, and realizes a reagentless glucose biosensing.

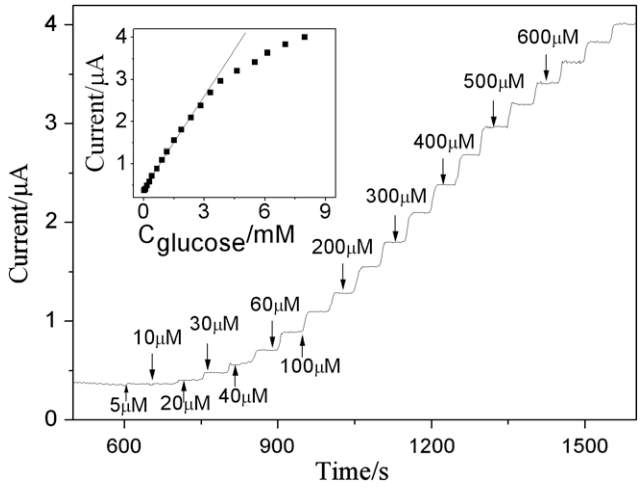

Fig. 5. Amperometric responses of GOD/MWNTs-Fc/CS nanocomposite-modified electrode at an applied potential of +350 mV to successive addition of glucose in a stirred 0.1 M PBS. Inset: calibration curve.

Fabrication reproducibility and storage stability of the biosensor

To prove the precision and practicability of the proposed method, the reproducibility and storage stability of the biosensor were examined. The relative standard deviation (RSD) of the modified electrode response to 0.1 mM glucose was 2.1% ($n = 5$). The RSD for five different electrodes using the same conditions response to glucose was 4.3%. These results showed that such kinds of electrodes exhibited good reproducibility. When the enzyme electrode was not in use, it was stored in PBS at 4 °C. After a storage period of more than 4 weeks the biosensor kept 91% of its initial response. The excellent analytical performance and long-term stability of the fabricated biosensor can be attributed to three aspects: First, biopolymer chitosan provides a biocompatible microenvironment around the enzyme molecules to stabilize its biological activity to a large extent. Second, the formation of the MWNTs-Fc conjugant prevents the Fc from leaking out of the film. Lastly, MWNTs provided a conduction pathway to accelerate electron transfer due to their excellent conductivity.

Study on the interference

High mediator concentrations have been reported to lessen the effect of oxygen on the electrode sensitivity [49]. One possible benefit of MWNTs-Fc conjugated is the alleviation of oxygen interference. Measurement of the glucose response under identical conditions (without degassing the solution) shows 6% loss of the sensitivity compared to that in the absence of oxygen. This is better than that reported for third-generation electrodes (e.g., 50% in

[49]). Interference experiments reveal that cysteine (0.1×10^{-3} M) and uric acid (0.1×10^{-3} M) do not give any obvious interference to 1.0×10^{-3} M glucose. Only ascorbic acid (0.1×10^{-3} M) causes significant interference because of its being oxidized at an applied potential of 350 mV, which can be avoided by coating a preselective film of Nafion polymer on the outside of the GOD/MWNTs-Fc/CS-modified electrode [48,50–53].

Conclusion

In conclusion, we have demonstrated that the MWNTs-Fc nanocomposite can be effectively employed for fabrication of the reagentless glucose biosensor. This composite not only provides a very suitable environment for enzyme entrapment but also establishes efficient electronic communication between GOD and the electrodes. The synergy of MWNTs and Fc in the MWNTs-Fc nanocomposites enhances the electrocatalytic action of Fc to the enzymatic oxidation of glucose by GOD and the electric communication between GOD and electrode, resulting in a sensitive amperometric sensor for glucose. The proposed reagentless glucose biosensor exhibits wide linear detection range, acceptable reproducibility, and operational and storage stability. We believe that the electron transfer mediator immobilized technique has promising perspectives for applications in the field of biosensor and bioelectronic devices.

Acknowledgments

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