Improvement of amperometric glucose biosensor by the immobilization of FcCD inclusive complex and carbon nanotube

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A novel glucose biosensor was constructed by using ferrocene-carbonyl-β-cyclodextrin (FcCD) inclusive complex as electron-transfer mediator and carbon nanotubes (CNTs) as electron-transfer promoter. FcCD inclusive complex and glucose oxidase (GOx) were covalently bonded to CNTs by poly-L-lysine (PLL) to fabricate a glucose biosensor. The electrocatalytic oxidation of glucose at the biosensor occurred at low potential below 200 mV, avoiding the interference of the main interfering substances in real samples containing a 5-times higher concentration of L-cysteine, ascorbic acid, and uric acid. The biosensor showed fast response for glucose. A broad linear range of glucose concentration from 1.0×10^{-5} to 2.90×10^{-3} M was obtained and the detection limit was 2.2×10^{-6} M.

1. Introduction

Rapid determination of blood sugar is important for the treatment and control of diabetes. Reagentless amperometric biosensors for glucose have attracted much interest for decades.[1–5] Most studies are based upon the use of the enzyme glucose oxidase (GOx) to catalyze the following reaction:

$$\text{glucose} + O_2 \rightarrow \text{gluconolactone} + H_2O_2$$

In this reaction, oxygen is used as the mediator to transfer electrons between the enzyme and the surface of electrode. However, the electrode response is very slow and is susceptible to the environmental oxygen concentration.

In most cases, direct electron transfer between the enzyme and the electrode is quite difficult, due to the deeply embedded redox center of the enzyme, which is surrounded by the thick protein layer. Therefore electron mediators other than oxygen for glucose oxidase are required to establish an electric connection between the redox center of enzyme and the electrode. Many mediators have been employed previously including hexacyanoferrate(III),[6–8] organic dyes,[9–11] Ru-complexes,[12] Os-complexes,[13–15] and other electrochemically active substrates.[16] Among the mediators, ferrocene (Fc) and its derivatives[17,18] were shown to be efficient electron acceptors in their oxidized ferricinium ion forms for soluble glucose oxidase.

Ferrocene is not readily entrapped at an electrode due to the high solubility of its oxidized ferricinium ion in aqueous solution, which makes it easy to diffuse away from the electrode surface into the bulk solution. The leakage of ferrocene/ferricinium mediators from the thin film of enzyme electrodes leads to their deterioration of sensitivity. Leakage can be avoided through the covalent bonding of glucose oxidase and ferrocene derivatives. Ferrocene has been functionalized with electron withdrawing groups (such as –COOH, –CHO, $-C(NOH)CH_3$),[19–21] which is covalently attached to the cyclopentadienyl ring of ferrocene. The electron withdrawing effect of the functional group decreased the electron cloud density of the cyclopentadienyl ring, which made it more difficult to be oxidized and the redox potentials of ferrocene derivatives were higher than that of ferrocene. It has been shown that the functionalized ferrocene derivatives accept electrons from GOx at relatively more positive redox potentials from 300 to 500 mV (*vs.* SCE), comparing with that of 165 mV of ferrocene. However, at higher potential, severe interference caused by endogenous electroactive species such as; ascorbic acid (AA), uric acid (UA), and cysteine (Cys) in blood samples can result in poor selectivity. Therefore, it is still a challenge to synthesize ferrocene derivatives with low redox potential.

Recently, carbon nanotubes (CNTs) have attracted much interest in various areas, such as biosensors and biofuel cells, due to their high surface area, good electrical conductivity, and significant mechanical strength.[22] It has been demonstrated that CNTs can promote direct electron transfer of redox enzymes.[23] Single-wall carbon nanotube[24] and multi-wall carbon nanotube[25] have been used to immobilize redox enzymes for the detection of H_2O_2.

In a previous paper, we synthesized a novel inclusive complex Fc-carbonyl-β-CD (FcCD). It showed that the redox reversibility and solubility of the FcCD complex were fairly good and can act as an electron transfer mediator and electrochemical probe.[26] However, the cavity of β-CD hindered the electron transfer between Fc and electrode surface, which made it 20 mV more positive than Fc. In the present paper, CNTs are applied to promote the electron transfer between FcCD complex and electrode surface. This amperometric biosensor based on immobilization of glucose oxidase and FcCD inclusive complex on CNTs was fabricated for the detection of glucose. The catalytic peak potential of the biosensor was shown at low potential. High

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concentration of known interferents did not influence the detection of glucose.

2. Experimental

2.1. Chemicals

β-cyclodextrin, H_2O_2, ferrocene, β-D-glucose, HNO_3, $NaBH_4$, and other reagents were all of analytical purity and purchased from Beijing Chemical Reagent Factory were used without further purification. CNTs were obtained from Shenzhen Nanotech Port (Shenzhen, China). Glucose oxidase (GOx, E.C. 1.1.3.4, 100 U mg^{-1}), Poly-L-lysine (PLL) hydrobromide (M_w = 30,000–70,000), *N*-hydroxysuccinimide (NHS) and 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma.

2.2. Preparation of carbonyl-β-CD and Fc-carbonyl-β-CD complex

The Fc-carbonyl-β-CD complex was synthesized according to the literature.[24]

In a typical procedure, β-CD (11.35 g, 0.01 mol) was added to a solution containing 10 ml 30% H_2O_2, 10 ml H_2O and 2 ml 1 M HNO_3 and then refluxed for 10 h. The milky solution became a transparent clear solution. After cooling, the reaction mixture was washed successively with acetone and anhydrous ethanol several times to remove the water. Then the solvent was removed under reduced pressure. The product carbonyl-β-CD was collected and dried overnight in a vacuum oven at 60 °C.

The inclusive complex was prepared by adding Fc into the carbonyl-β-CD solution (1 : 1 molar ratio). After stirring at room temperature for 10 min, the mixed solution was transferred into a Teflon-lined stainless autoclave at 100 °C for 12 h. After cooling, the mixed solution turned to yellow transparent solution. The reaction mixture was washed successively with acetone and anhydrous ethanol several times to remove the water. Then the solvent was removed under reduced pressure. The product Fc-carbonyl-β-CD complex was collected and dried overnight in a vacuum oven at 60 °C.

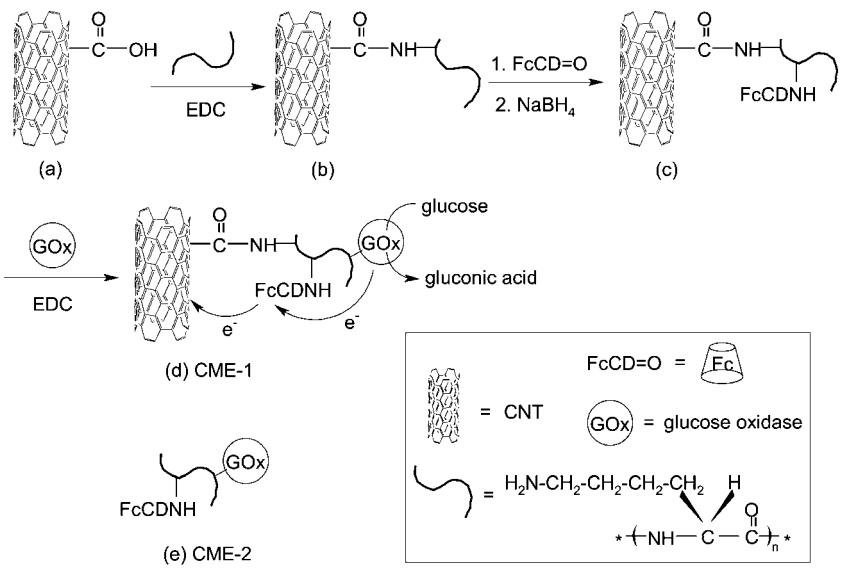
2.3. Preparation of CNT-PLL-FcCD-GOx and PLL-FcCD-GOx

Before use, CNTs were dispersed in 30% HNO_3 and then refluxed for 24 h at 140 °C to obtain carboxylic group-functionalized CNTs. The resulting suspension was centrifuged, and washed with deionized water until the pH reached 6.0, and dried in a vacuum. Generally, CNT-PLL was prepared by vigorously stirring a 2.0 ml PBS solution containing 10 mg CNT, 4 mg PLL, 2 mg NHS and 2 mg EDC at 50 °C for 24 h. After that, the CNT-PLL was collected by centrifugation, thoroughly washed with alcohol and deionized water, and dried in a vacuum, to remove any unreacted PLL and EDC.

Then, 2 mg Fc-carbonyl-β-CD complex was dissolved in 2.0 ml CNT-PLL/PBS solution. The solution was reacted at 60 °C for 12 h to form a Schiff-base, and then an excess of $NaBH_4$ was added to reduce the Schiff-base to an amine. The reduction was left for another 6 h to ensure complete reaction. The CNT-PLL-FcCD was collected by centrifugation, washed with deionized water and ethanol several times to remove the unreacted reactants and other reagents, and then dried at 60 °C for 4 h in a vacuum.

Finally, 2 mg glucose oxidase and 2 mg EDC were dissolved in 2.0 ml CNT-PLL-FcCD/PBS solution and stirred at room temperature for 24 h. The product was collected by centrifugation, washed with deionized water several times. And then dissolved in 2.0 ml PBS solution and stored at 4 °C in a refrigerator. Thus, CNT-PLL-FcCD-GOx (CME-1, Scheme 1d) was obtained.

The reference PLL-FcCD-GOx without CNT (CME-2, Scheme 1e) was prepared in the same way except that PLL was used instead of CNT-PLL. In each step of the purification, the solution was subjected in a dialysis bag (12 kDa) for 2 days with regular change of water every 12 h. After purification, the



Scheme 1 Illustration of the process for preparation of CNT-PLL-FcCD-GOx modified electrode.

solution was frozen and dried to solid powder. The product was then dissolved in 2.0 ml PBS solution and stored at 4 °C in a refrigerator.

2.4. Preparation of CNT-PLL-FcCD-GOx and PLL-FcCD-GOx modified electrodes

Prior to each experiment, glassy carbon electrode was polished successively with 1, 0.3 and 0.05 μm α-alumina powder, rinsed thoroughly with doubly distilled water between each polishing step, sonicated in 1 : 1 nitric acid : acetone and doubly distilled water successively, and then allowed to dry at room temperature.

CNT-PLL-FcCD-GOx electrode was prepared in the following procedure: 1.0 ml CNT-PLL-FcCD-GOx/PBS solution was mixed with 10 μl Nafion ethanol solution (5%, Aldrich) and sonicated for 30 min. Then, 10 μl of the mixed solution was applied to the working electrode surface by a micropipette to form a thin layer and stored at 4 °C in dry state.

PLL-FcCD-GOx electrode was prepared in the same way of CNT-PLL-FcCD-GOx electrode.

Fc/GOx electrode was prepared in the following procedure: First, 1 mg Fc was dissolved in 1.0 ml absolute ethanol. 10 μl of Fc solution was cast on the working electrode surface and allowed to dry in the air. Then 1 mg glucose oxidase/1.0 ml PBS solution was mixed with 10 μl of Nafion ethanol solution. 10 μl of the mixed solution was applied to the working electrode surface by a micropipette to form a thin layer and stored at 4 °C in dry state.

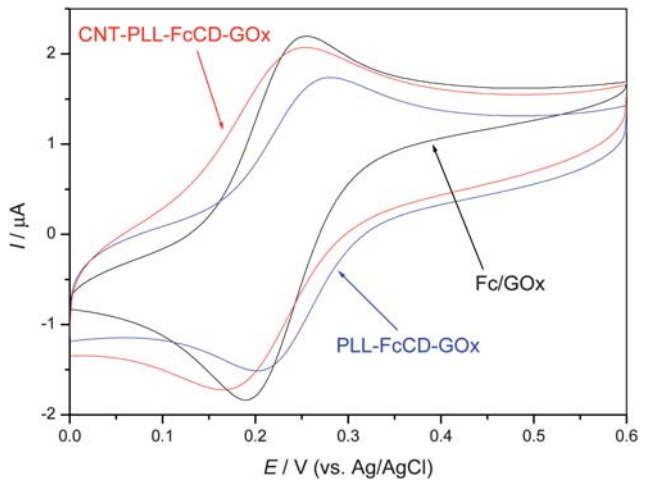


Fig. 1 Cyclic voltammograms of CNT-PLL-FcCD-GOx, PLL-FcCD-GOx and Fc/GOx modified electrode in 0.1 M PBS (pH = 6.5). Scan rate 100 mV s^{-1}.

2.5. Apparatus

Cyclic voltammetry and chronoamperometric measurements were performed by CHI 660A electrochemical workstation (Chenhua, China). Glassy carbon electrode (GCE) (3 mm diameter) was used as the working electrode, a platinum plate (Pt) as the counter electrode and a Ag/AgCl (saturated KCl) electrode as the reference electrode.

3. Results and discussion

3.1. Preparation of glucose biosensors

The biosensor film was prepared as illustrated in Scheme 1. Firstly, carboxylated CNTs reacted with poly-L-lysine to form CNTs with amine groups. Then, carbonyl group of the Fc-carbonyl-β-CD complex reacted with the amine group of CNTs to form a Schiff's base. The Schiff's base was then reduced to an amine by $NaBH_4$. In this method, the Fc-carbonyl-β-CD complex was covalently bonded to the CNTs. Then glucose oxidase was attached to CNTs by EDC reagent. The CNT-PLL-FcCD-GOx was dispersed onto the electrode surface to form glucose biosensor film.

3.2. Electrochemical studies of the biosensors

Fig. 1 shows the cyclic voltammogram of CNT-PLL-FcCD-GOx, compared with that of PLL-FcCD-GOx and Fc/GOx on glassy carbon electrodes. The redox potential of Fc/GOx is 0.26/0.19 V (*vs.* Ag/AgCl). When Fc is included into the cavity of β-CD, the redox potential of PLL-FcCD-GOx shifted positively to 0.28/0.21 V (*vs.* Ag/AgCl), indicating that the cavity of β-CD hindered electron transfer between the electrode surface and Fc. However, when PLL-FcCD-GOx was attached to CNTs, the redox potential shifted negatively to 0.25/0.17 V (*vs.* Ag/AgCl). It showed that CNTs promote electron transfer between the electrode surface and FcCD.

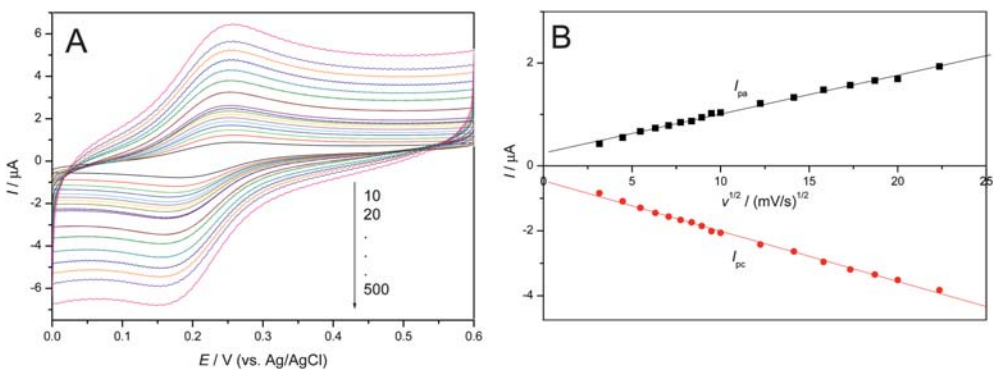


Fig. 2 (A) Cyclic voltammograms of the biosensor in 0.1 M PBS (pH = 6.5). Scan rates are 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, and 500 mV s^{-1} (from inner to outer), respectively. (B) Dependence of anodic and cathodic peak currents with the square root of scan rate.

Fig. 2A shows cyclic voltammograms of CNT-PLL-FcCD-GOx in 0.1 M PBS (pH 6.5) solution at different scan rates. Redox peaks at 0.17 and 0.25 V (*vs.* Ag/AgCl) were seen, which can be attributed to FcCD. The $\triangle E_p$ is 80 mV and did not change with increasing scan rate, indicating that the mediator underwent a quasi-reversible redox reaction. The anodic and cathodic peak currents increased linearly with the square root of scan rate from 10 to 500 mV s^{-1} (Fig. 2B), indicating a diffusion-like behavior by electron-hopping and charge motion.

3.3. Electrochemical oxidation of glucose on the biosensor

Fig. 3 compares cyclic voltammograms of CNT-PLL-FcCD-GOx with those of PLL-FcCD-GOx in 0.1 M PBS solutions with different concentration of glucose. In the absence of glucose, the GOx gave no response and only redox peaks of FcCD were observed. When the substrate glucose was added to the electrolyte solution, a remarkable increase of catalytic current was observed, indicating that FcCD inclusive complexes acted as electron mediators between the electrode substrate and the redox center of GOx. The response current of 5.0 mM glucose at CNT-PLL-FcCD-GOx is 8.46 μA, which is 40% higher than that at PLL-FcCD-GOx (6.04 μA). The catalytic peak potential of CNT-PLL-FcCD-GOx is 30 mV more negative than that of PLL-FcCD-GOx. The results indicated that CNTs promoted the electron transfer between FcCD and electrode surface.

3.4. Effect of the applied potential, pH, and temperature on the response of biosensor

The effect of applied potential on the response of the glucose was investigated from 0 to 500 mV (*vs.* Ag/AgCl). The glucose electrocatalytic oxidation current increased rapidly under the potential from 0 to 200 mV, and reached a plateau at 200 mV. Further increase of potential from 200 to 500 mV did not increase the response current much. In order to obtain high response current and avoid the interfering of the interference substances, the electrode potential was maintained at 200 mV (*vs.* Ag/AgCl) for amperometric measurements.

It is well known that both the diffusion coefficient of the mediator and the activity of GOx were affected by pH value. The pH dependence of the biosensor response was evaluated over the pH range from 6.0 to 8.0. The biosensor displayed an optimum sensitivity of the response at pH 6.5.

Temperature played an important role on the enzyme activity and catalytic reaction. The effect of temperature on the sensor was investigated between 10 and 50 °C. An increase of temperature enhanced the sensitivity of the biosensor to glucose until it reached a maximum value at 40 °C. Further increase of temperature led to a decrease of the sensitivity of the biosensor, which should be due to the partial denaturation of the enzyme. Taking lifetime and response characteristics into consideration, 25 °C was selected as the work temperature.

3.5. Steady-state current measurement to glucose

Fig. 4 shows I-t curves of glucose response in the three different electrodes. There was no response at the GOx/GC electrode. When Fc was added to the GOx film, the GOx/Fc/GC electrode showed an obvious response of glucose. However, the response current was small and decreased with the time of potential applied due to the diffusion of oxidised Fc (Fc^+) from the electrode film to the solution. When Fc was included into the cavity of β-CD and attached to the film, the response current was enhanced and did not show any decay. When the FcCD was attached to CNTs and then cast on the electrode surface, the response current was further enhanced. It indicated that CNTs promoted the electron transfer between FcCD and electrode surface.

The electrocatalytic oxidation of glucose on GOx-FcCD-PLL-CNT/GC electrode was studied by amperometry. Fig. 5A showed the typical steady state current-time response of the biosensor. The electrode response time was less than 5 s until steady state values were obtained. The fast response was attributed to the thin active film and short penetration depth of the

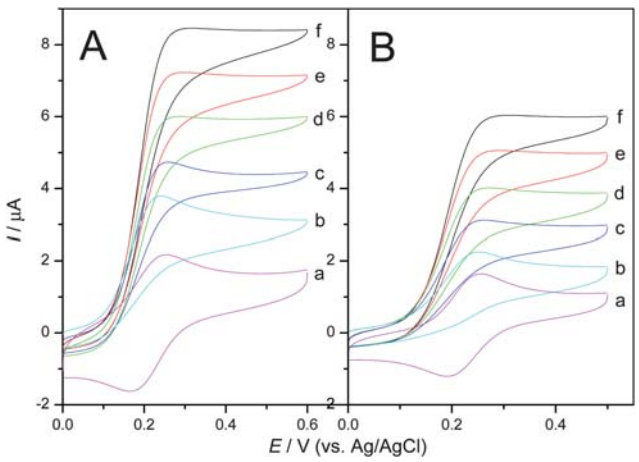


Fig. 3 Cyclic voltammograms of CNT-PLL-FcCD-GOx (A) and PLL-FcCD-GOx (B) taken in deaerated PBS (pH = 6.5) in the absence (a) and presence of 1.0 (b), 2.0 (c), 3.0 (d), 4.0 (e), 5.0 (f) mM glucose. Scan rate: 100 mV s^{-1}.

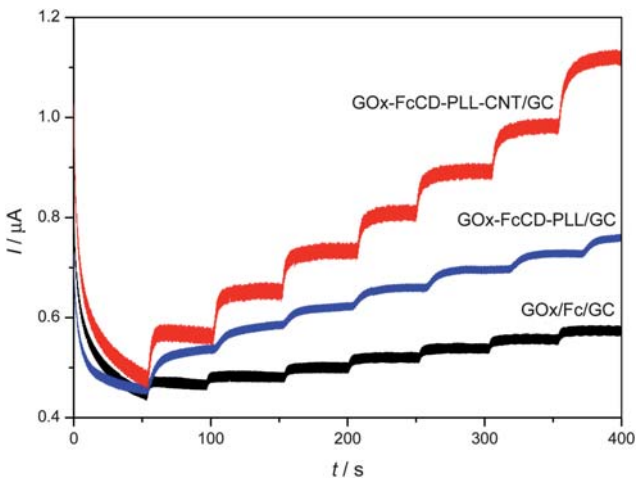


Fig. 4 Chronoamperometric responses of 0.05 mM glucose at GOx/Fc/GC, GOx-FcCD-PLL/GC, and GOx-FcCD-PLL-CNT/GC electrodes. Potential applied: 0.20 V (*vs.* Ag/AgCl).

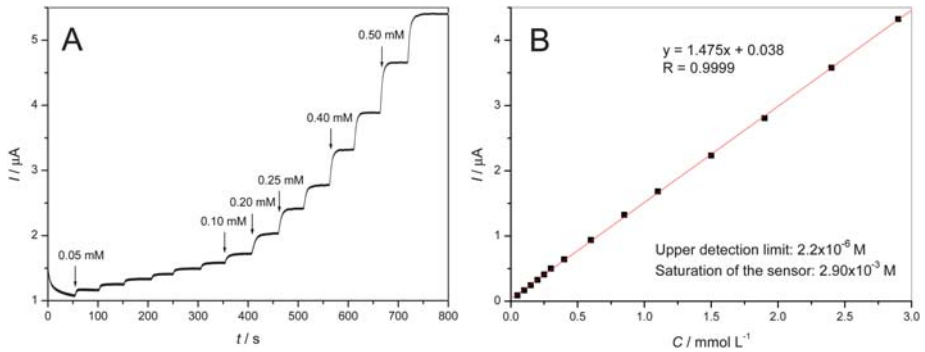


Fig. 5 (A) The chronoamperometric responses to glucose additions obtained in 0.1 M PBS. Potential applied: 0.20 V (*vs.* Ag/AgCl). (B) The calibration curve of the oxidation currents on the concentration of glucose.

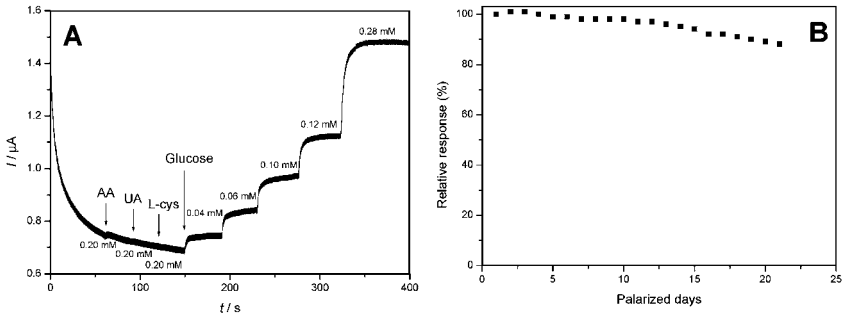


Fig. 6 (A) The amperometric response of 0.20 mM ascorbic acid, uric acid, L-cysteine, and different concentration of glucose on the biosensor. (B) Storage stability of the GOx-FcCD-PLL-CNT/GC electrode. Polarization potential: 0.2 V (*vs.* Ag/AgCl). Responses of 0.05 mM glucose were compared.

electrode. The calibration curve gave a linear range of the concentration of glucose from 1.0×10^{-5} to 2.90×10^{-3} M with a correlation coefficient of 0.9999 (Fig. 5B). The detection limit was calculated 2.2×10^{-6} M (S/N = 3).

3.6. Interferences studies and stability of GOx-FcCD-PLL-CNT/GC biosensor

Interferences effects were investigated in Fig. 6A by testing the response of the biosensor to L-cysteine (L-cys), ascorbic acid (AA), and uric acid (UA). The three compounds are the main substances that interfere with the detection of glucose in blood and urine samples. It shows that a 5-times higher abundance of the three interferents did not affect the measurement of glucose. Therefore the biosensor with low redox potential using FcCD inclusive complex as mediator and CNT as electron transfer promoter avoids the interference problem effectively.

The reproducibility of the biosensor was examined by applying 8 electrodes in 0.2 mM glucose solution for measurements. The relative standard derivation value was 2.8%. And the repeatability of one electrode was also examined by repeated measurements. The catalytic oxidation current decreased only 4.9% after 200 successive measurements. The storage stability of one biosensor was studied over a period of 21 days (Fig. 6B). By storing it as a dry film in the refrigerator at 4 °C and measuring once a day, the biosensor performed well over 3 weeks, at the end of which time it maintained 88% of its initial response.

4. Conclusions

In summary, Fc-carbonyl-β-CD inclusive complex and CNTs were used to prepare a reagentless amperometric glucose biosensor. The biosensor shows fast response for the analyte and a broad linear range with low detection limit. It also exhibits excellent sensitivity in the presence of a 5-times higher concentration of known interferents; L-cysteine, ascorbic acid, and uric acid without significant affect on the measurement of glucose.

Acknowledgements

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