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A selective novel non-enzyme glucose amperometric biosensor based on lectin–sugar binding on thionine modified electrode

Feng Li[a,b], Yan Feng[b], Limin Yang[b], Liang Li[b], Chenfei Tang[b], Bo Tang[a,*]

[a] College of Chemistry, Chemical Engineering and Materials Science, Engineering Research Center of Pesticide and Medicine Intermediate Clean Production, Ministry of Education, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Shandong Normal University, 88 Wenhua East Road, Jinan 250014, People's Republic of China
[b] State Key Laboratory Base of Eco-Chemical Engineering, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, People's Republic of China

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ABSTRACT

A novel non-enzyme glucose amperometric biosensor was fabricated based on biospecific binding affinity of concanavalin A (Con A) for D-glucose on thionine (TH) modified electrode. TH can be covalently immobilized on potentiostatically activated glassy carbon electrode through Schiff-base reaction. Subsequently, the surface-adherent polydopamine film formed by self-polymerization of dopamine attached to TH and afforded binding sites for the subsequent immobilization of Con A molecules via Michael addition and/or Schiff-base reaction with high stability. Thus, a sensing platform for specific detection towards D-glucose was established. The binding of Con A towards D-glucose can be monitored through the decrease of the electrode response of the TH moiety. Due to the high affinity of Con A for D-glucose and high stability of the resulting sensing platform, the fabricated biosensor exhibited high selectivity, good sensitivity, and wide linear range from 1.0×10^{-6} to 1.0×10^{-4} M with a low detection limit of 7.5×10^{-7} M towards D-glucose.

1. Introduction

Diabetes mellitus which is a leading cause of death and disability nowadays has become a public health concern. Patients with diabetes are suffering from metabolic disorder, also exposed to significant long-term risks of complications, including heart disease, kidney failure, and blindness. According to population studies, diabetes mellitus has affected approximately 171 million individuals worldwide in the year 2000 and it will reach up to 366 million by the year 2030 with an exponential prevalence (Wild et al., 2004). Therefore, the determination of blood glucose level is of great of significance for the diagnosis and management of diabetes mellitus (Pickup et al., 2005).

Numerous glucose biosensors were developed for glucose measurement since the first glucose biosensor was developed. Turbidity sensors (Ballerstadt et al., 2007), viscometric affinity sensors (Diem et al., 2004), surface plasmon resonance based sensors (Gallego et al., 2004), fluorescence based sensors (Russell and Pishko, 1999), and electrochemical biosensors (Li et al., 2009a) have been extensively studied. Among them, the electrochemical biosensor can be a good choice for its simplicity, rapid response, high sensitivity, and low cost. As a member of electrochemical biosensors, enzymatic glucose electrochemical biosensors based on modification of glucose oxidase on various substrates are of particular importance due to the biospecific affinity of enzyme for its substrate. However, friendly environment is essentially required to retain the activity of the enzyme (Li et al., 2009b), leading to a higher requirement on material selection and operation conditions. Furthermore, there is severe interference caused by endogenous electroactive uric acid (UA) and ascorbic acid (AA) in blood samples (Safavi et al., 2008). The two points above limit the application of enzymatic biosensors in a way. As a result, non-enzyme based glucose biosensors have been receiving increased attention in recent years (Aslan et al., 2004; Ma et al., 2009).

Usually, non-enzyme based glucose biosensors are fabricated based on the specific affinity of certain species for glucose, including boronic acid derivatives (Yu and Yam, 2009), antibodies (Engstrőm et al., 2008), and lectins (Nakata et al., 2005). Concanavalin A (Con A), one member of lectins family, has been extensively used in glucose recognition. It appears to be dimeric below pH 5.5, and undergoes the dimer–tetramer equilibrium between 5.5 and 7.5. At pH > 7.5, the protein exists as a tetramer (molecular mass, 104,000 for tetramer) (Seneart and Teller, 1981). Under neutral conditions, each subunit of Con A contains one binding site for D-mannose and D-glucose residues; one for calcium and manganese cations, which activate the binding site of protein for carbohydrates; the third one for hydrophobic recognition (Liu et al., 2007). Glucose measurements based on Con A–glucose binding were commonly

* Corresponding author. Fax: +86 531 86180017.
E-mail address: tangb@sdnu.edu.cn (B. Tang).

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conducted by fluorescence biosensors, due to its intrinsic high sensitivity, however, the sensor performance was often influenced by non-specific changes in fluorescence (Labib et al., 2010). Alternatively, it will be an efficient and reliable way to apply Con A–glucose binding to the fabrication of glucose amperometric biosensor. To the best of our knowledge, there is no publication on the usage of Con A–glucose binding for fabrication glucose amperometric biosensor yet.

The efficient immobilization of Con A on underlying substrates is always a challenge, as Con A will denature and loose activity when it is adsorbed on bare surfaces. Dopamine (3,4-dihydroxyphenylethylamine) is an analogue of 3,4-dihydroxyphenylalanine which plays a major role in strong adhesion of a mussel onto a variety of substrates in tidal environment (Lee et al., 2008a). In the presence of dissolved O_2 and under alkaline conditions, aqueous solutions of dopamine will spontaneously oxidize in about 10 min to polydopamine (pDA) which is an adherent polymer coating on both organic and inorganic surfaces (Lee et al., 2008b). The DA self-polymerization mechanism was not clear until now. It is acceptable that DA is firstly oxidized to dopaminequinone; the intramolecular cyclization of dopaminequinone via 1,4-Michael addition leads to oxidizable leucodopaminechrome; then leucodopaminechrome is oxidized to dopaminechrome. Dopaminechrome can further undergo polymerization reactions, yielding deposited melanin-like polymer (Li et al., 2006). The resulting pDA film can be an appealing material for immobilization of Con A through Michael addition and/or Schiff-base reaction between the quinone/catechol groups of pDA and the ε-NH_2 of the lysine residues of Con A (Morris et al., 2009).

Herein, a novel selective non-enzyme based glucose amperometric biosensor was fabricated based on the specific affinity of Con A for D-glucose. TH acted as the electrochemical response indicator, which was covalently immobilized on potentiostatically activated glassy carbon electrode (GCE). Then, pDA was formed on TH modified surface as an adhesive coating in alkaline solution, which also afforded binding sites for the adhesion of Con A. The binding between Con A and D-glucose was monitored through the decrease of the electrochemical response of the TH moiety. The stability of the sensing platform, the high affinity of Con A for D-glucose as well as the excellent electron transfer ability of TH endowed the as-prepared glucose biosensor with low detection limit, relatively wide linear range, good reproducibility, long-term stability and high anti-interference ability.

2. Experimental

2.1. Chemicals

Dopamine hydrochloride, dextran (39 kDa) and D-glucose were purchased from Sigma–Aldrich and used as received. TH was purchased from Guo Yao Company (China). Con A (Shanghai sanjie biotechnology Co. Ltd.) from canavalin ensiformis (Jack Bean) was used in this study. The 0.1 M phosphate buffer solutions (PBS) at various pH values were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4, and adjusting the pH with 0.1 M NaOH or H_3PO_4. All other chemicals were of analytical grade and doubly-distilled water (DDW) was used throughout this work.

2.2. Apparatus and instruments

Cyclic voltammetric (CV) and differential pulse voltammetric (DPV) measurements were performed on a CHI 832B electrochemical analyzer (Shanghai CH Instrument Company, China). Electrochemical impedance spectroscopy (EIS) was performed on a CHI 660C electrochemical analyzer (Shanghai CH Instrument Company, China). A conventional three-electrode system was used with bare GCE or modified GCE as the working electrode, Ag/AgCl electrode (saturated with KCl) as the reference electrode, and platinum wire as the auxiliary electrode, respectively.

2.3. Preparation of pDA film on TH modified GCE

GCE was used as the substrate electrode in the construction of the biosensor. Prior to use, the bare GCE was polished with 1.0, 0.3, and 0.05 μm alumina slurry to obtain a mirror like surface, respectively, followed by thorough rinsing with DDW, and sonicating in DDW and ethanol. Potentiostatic activation was carried out by oxidizing the polished electrodes in 0.1 M HNO_3 at 1.8 V for 3 min, and then reducing at −1.5 V for 1 min (Chen et al., 2009). For covalent immobilization of TH, the obtained electrode above was immersed in 2 mg/mL TH solution (0.1 M PBS pH 7.0) for 3 h, and then washed by ultrasonication for 10 min, followed by rinsing with 0.1 M PBS (pH 7.0) to remove non-specifically adsorbed TH. The as-prepared electrode was denoted as TH/GCE.

Subsequently, the TH/GCE was immersed into 6 mM dopamine solution (0.1 M PBS pH 8.5) for 2 h under stirring (Lee et al., 2007). Then, the electrode was thoroughly rinsed with DDW and dried with nitrogen, and denoted as pDA/TH/GCE.

2.4. Immobilization of Con A on pDA modified surface and the binding of D-glucose

10 mg Con A was dissolved in 10 mL 0.1 M PBS (pH 7.0) containing 0.1 M $CaCl_2$ and 0.1 M $MnCl_2$ and kept for 12 h at 4 °C to obtain 1 mg/mL Con A solution. The immobilization of Con A was performed by incubating pDA modified electrode in 1 mg/mL Con A solution under stirring for 2 h at room temperature (Morris et al., 2009). After the immobilization step, the electrode was carefully rinsed with 0.1 M PBS (pH 7.0) and Con A/pDA/TH/GCE was thus obtained. To examine the interaction between D-glucose and Con A, the Con A/pDA/TH/GCE was incubated in 0.1 M PBS (pH 7.0) containing various concentrations of D-glucose under stirring. The incubation time of Con A binding for D-glucose was also investigated.

2.5. Characterization of the developed biosensor

The modified electrodes obtained during stepwise modification were electrochemically characterized by using CV, EIS and DPV. CV and DPV measurements were performed in 0.1 M PBS (pH 7.0) quiescent solutions with a scan rate of 50 mV s^{-1}. EIS measurement was performed with the frequency ranging from 10^4 to 10^{-1} Hz in 0.1 M KCl containing 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) with the AC voltage amplitude was 5 mV, and the applied potential of 221 mV versus Ag/AgCl electrode.

3. Results and discussion

3.1. Immobilization of Con A on pDA modified electrode and the binding of D-glucose

As is known to all, Con A has specific affinity for D-glucose and D-mannose, based on which a novel glucose amperometric biosensor was constructed for selective recognition of D-glucose. As is illustrated in Fig. 1, TH was firstly immobilized on CHO-riched surface which was generated via a potentiostatic activation process in diluted nitric acid. Potentiostatically activated GCE surface possessed microporous structure (Nagaoka and Yoshino, 1986), leading to an increased electrode area available for loading of TH. The in situ generated-COH reacted with TH to form imine bond, which contributed to the stable attachment of TH on electrode

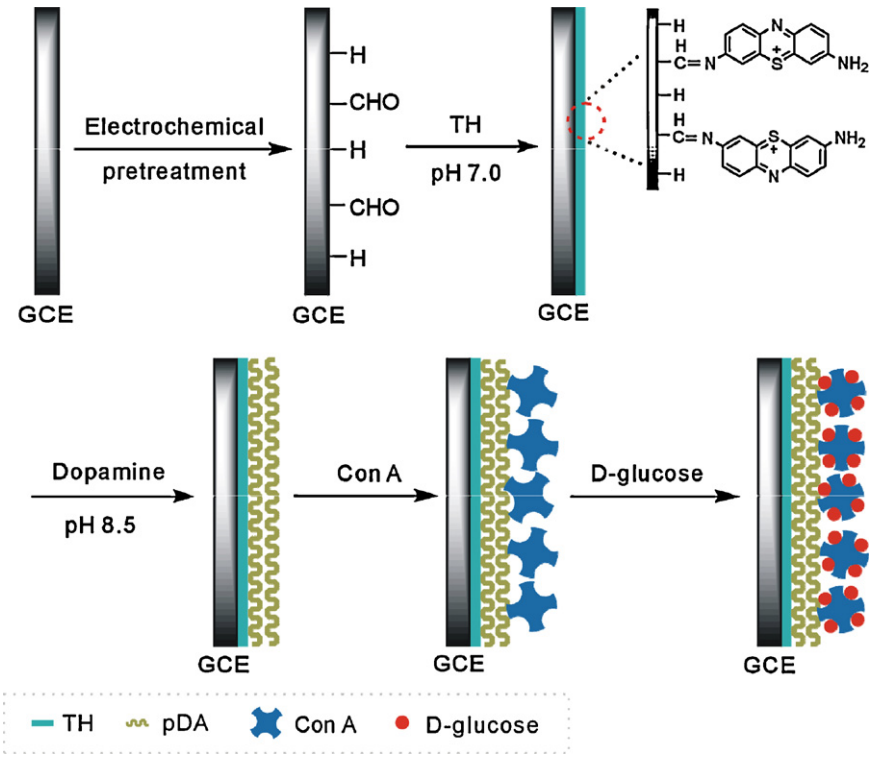

Fig. 1. Schematic presentation of the fabricated glucose amperometric biosensor.

surface. Then, pDA adhesion layer was formed on the amine-terminated electrode surface just by immersion of the electrode in dopamine alkaline solution (Lee et al., 2007). It can be seen that the resulting electrode surface was covered by a brown-colored melanin-like polymer, indicating the successful formation of pDA film. Afterwards, Con A was covalently adhered to the pDA film through Michael addition and/or Schiff-base reactions. In fact, the nature of the interaction between the bound Con A and pDA is not quite clear. It was acceptable that the N atom of ε-NH_2 on the lysine residues of Con A owns lone pair electrons, thus –NH_2 can nucleophilically attack the α-carbon of an α,β-unsaturated quinone moiety of the pDA polymer, yielding amine–quinone adducts. Alternatively, –NH_2 can nucleophilically attack the C atom of the unsaturated carbonyl on the quinine moiety, leading to carbon–nitrogen double bonds between Con A and pDA (Morris et al., 2009; Burzio and Waite, 2000). The pDA adhesive coating had the stability similar to chemical bond coating (Yin and Liu, 2008), which can dramatically improve stability of TH layer as well as Con A on electrode surface, leading to enhanced stability of the sensing surface. The resulting sensing platform was thus constructed for specific determination of D-glucose.

3.2. Electrochemical behaviors of the glucose biosensor

Fig. 2 shows CVs of (a) bare GCE, (b) TH/GCE, (c) pDA/TH/GCE, (d) Con A/pDA/TH/GCE, and (e) glucose/Con A/pDA/TH/GCE in 0.1 M PBS (pH 7.0) over the potential range from 0.1 to −0.6 V at a scan rate of 50 mV s^{-1}. No redox peak appeared on the bare GCE (Fig. 2a), while a pair of well-defined redox peaks was observed at TH/GCE (Fig. 2b), with the anodic (E_{pa}) and cathodic potential (E_{pc}) of −0.22 V and −0.26 V, respectively. This can be undoubtedly attributed to the redox reaction of TH, indicating TH has been successfully immobilized on electrode surface. After pDA layer was formed on the TH/GCE via a spontaneous oxidization coating technique, the signal of the peak currents decreased, and the peak-to-peak separation (ΔE_p) was increased (Fig. 2c), which was probably that the pDA film had obstructed the electron transfer. Another oxidation peak located at ca. −0.04 V can be ascribed to the oxidation of leucodopaminechrome. When the Con A molecules were immobilized onto the pDA/TH/GCE (Fig. 2d) and the subsequent binding for D-glucose (Fig. 2e), the peak current further decreased, implying the attachment of Con A and glucose has enhanced the inhibition effect on the electron transfer. It has to be mentioned that the modified GCE in neutral solutions is quite stable during repetitive CV scans over the potential range of 0.1 to −0.6 V, and the current obtained on the as-prepared GCE suffers from almost no degradation. It is probable that pDA has stabilization effect on the sensing surface, which efficiently avoids the dissociation of TH from the electrode surface.

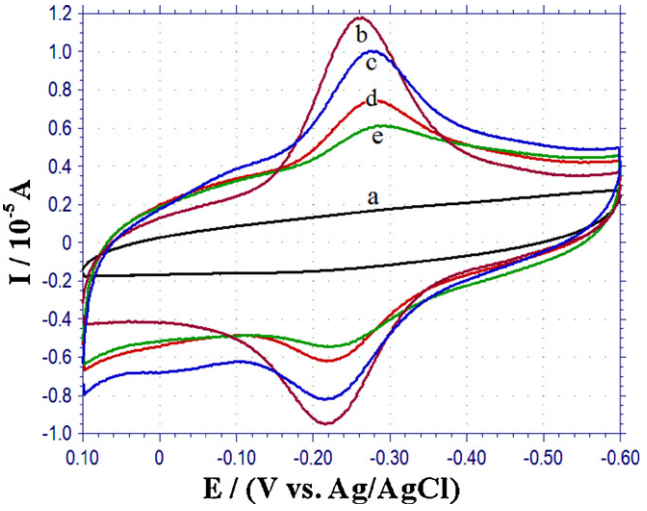

Fig. 2. CVs (a) bare GCE, (b) Th/GCE, (c) pDA/Th/GCE, (d) Con A/pDA/Th/GCE, and (e) glucose/Con A/pDA/Th/GCE in 0.1 M PBS (pH 7.0) at a scan rate of 50 mV s^{-1}; concentration of glucose: 4.0×10^{-5} M.

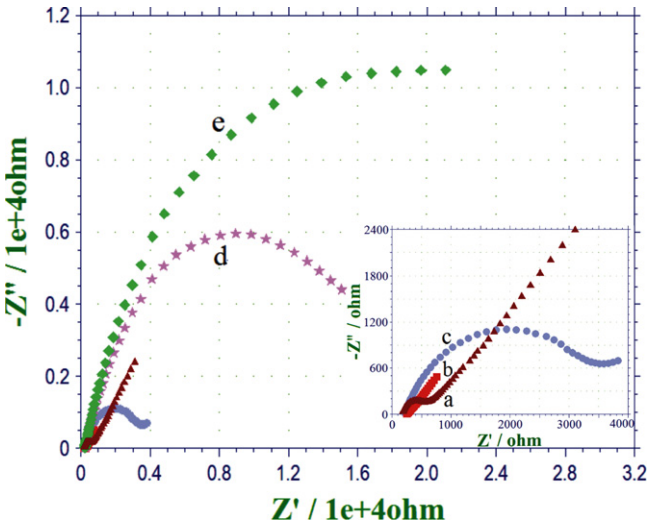

Fig. 3. EIS for (a) bare GC electrode, (b) Th/GCE, (c) pDA/Th/GCE, (d) Con A/pDA/Th/GCE, and (e) glucose/Con A/pDA/Th/GCE in 0.1 M KCl containing 5.0 mM $Fe(CN)_6^{3-}$ and 5.0 mM $Fe(CN)_6^{4-}$.

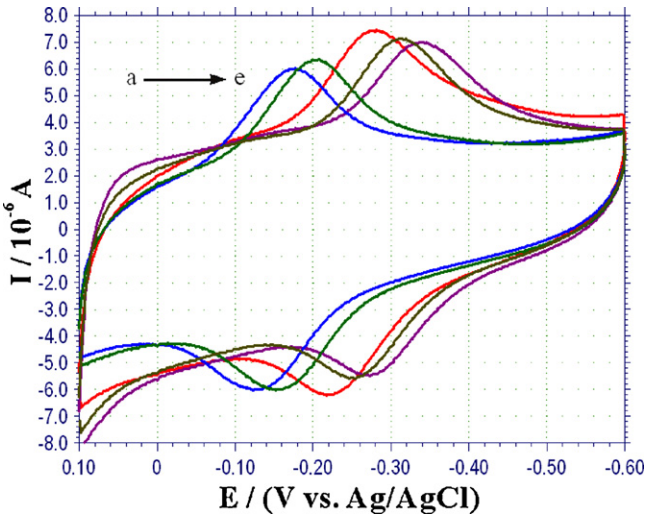

Fig. 4. CVs of the Con A/pDA/Th/GCE in 0.1 M PBS at various pHs: (a) 5.0, (b) 6.0, (c) 7.0, (d) 8.0, and (e) 9.0 at a scan rate of 50 mV s^{-1}.

Further study was performed to investigate the effect of scan rate on the response of TH at the Con A/pDA/TH/GCE in 0.1 M PBS (pH 7.0) (see supplementary material Fig. S1). CVs of Con A/pDA/TH/GCE gave nearly symmetric anodic and cathodic peaks. With the increasing scan rate, E_{pa} and E_{pc} were shifted slightly towards the positive and the negative direction of potential, respectively. Both the anodic and the cathodic peak currents of the Con A/pDA/TH/GCE were proportional to the scan rate between 20 and 500 mV s^{-1}, indicating a surface-controlled electrode process (Gosser, 1993).

EIS was employed to characterize the interface properties of the modified electrodes. Fig. 3 shows the typical results of AC impedance spectra of the bare GCE (a), TH/GCE (b), pDA/TH/GCE (c), Con A/pDA/TH/GCE (d), and glucose/Con A/pDA/TH/GCE (e) obtained in 0.1 M KCl containing 5.0 mM $Fe(CN)_6^{3-}$ and 5.0 mM $Fe(CN)_6^{4-}$ with the frequency ranging from 10^4 to 10^{-1} Hz. Significant differences in the electron transfer resistance (R_{et}) were observed upon the stepwise modification of the electrode. R_{et} valve of the bare GCE (Fig. 3a) was estimated to be 504 Ω. After covalent immobilization of TH onto the surface of pretreated GCE, the value of R_{et} was decreased noticeably (Fig. 3b), which is attributed to the strong electrostatic adsorption between the negatively charged $Fe(CN)_6^{3-/4-}$ and the positively charged TH molecules. The coating of pDA film on the surface of TH/GCE induced the R_{et} to be 3261 Ω (Fig. 3c), implying that the pDA film could largely restrict the electron transfer from the electrochemical probe towards electrode surface. The immobilization of Con A molecules onto the pDA film also led to an increased R_{et} valve (Fig. 3d), and the binding of glucose made the R_{et} valve even larger (Fig. 3e), indicating both large molecule Con A and the binding of glucose can obstruct the redox probe approaching towards the electrode surface.

3.3. Influence of pH on the biosensor responses

TH is a pH-dependent dye (Nicotra et al., 2008), so the electrochemical behavior of TH can be significantly influenced by the pH of supporting solutions. Fig. 4 shows CVs of Con A/pDA/TH/GCE in 0.1 M PBS from pH 5.0 to 9.0 at a scan rate of 50 mV s^{-1}. It is worthy to note that stable CV curves were obtained during successive scanning cycles in supporting solutions of different pH, suggesting that the proposed biosensor can be used over a wide pH range. It was found that both E_{pa} and E_{pc} shifted to a negative direction with the increase of pH, indicating a both proton and electron transfer process (Xu et al., 1998). The ΔE_p changed slightly, indicating a good reversibility of TH on the well-confined surface over the wide pH range. It can be seen that the maximum response of TH at the Con A/pDA/TH/GCE was obtained at pH 7.0. Taking account of the peak current response, the activity of the protein and condition of specific binding affinity of Con A for glucose (Sugawara et al., 2004), pH 7.0 was selected for further experiments.

3.4. Determination of D-glucose

Herein, a Con A/TH modified GCE was constructed for specific determination of D-glucose. When Con A modified electrode was immersed in D-glucose solution, the TH moiety was held at the binding site of Con A (Sugawara et al., 2004). The introduction of glucose into the binding site of Con A makes it difficult for the electron transfer between the redox TH and the electrode surface, which can be reflected by the decreased amperometric response of TH.

The incubation time of Con A modified electrode in D-glucose solution can influence the electrochemical response of the biosensor. In the experiment, different current signals of TH were obtained by immersion of the Con A/pDA/TH/GCE in 4.0×10^{-5} M D-glucose for 20, 40, 60, 80, and 100 min, respectively (data not shown for brief). With prolonging the incubation time to 60 min, the peak current change versus that of Con A/pDA/TH/GCE increased, and the peak current valve trended to almost a constant from 60 to 100 min. Thus, an incubation time of 60 min for Con A modified electrode immersion in D-glucose solution was chosen for experiment.

Fig. 5 shows DPV responses of Con A/pDA/TH/GCE to different concentrations of D-glucose by immersion of the modified electrode in D-glucose solution (0.1 M PBS pH 7.0) for 60 min before measurement. It can be found that each oxidation wave for the TH moiety was a well-defined peak and the oxidation current decreased continuously as a function of glucose concentration in the range of 1.0×10^{-6} to 1.0×10^{-4} M (shown in inset). The regression equation was expressed as ΔI_{pa} (10^{-6} A) = $-0.04279\,C$ (10^{-6} M) $- 0.42308$ ($n = 8$), with a correlation coefficient of −0.9969. A detection limit of 7.5×10^{-7} M D-glucose can be estimated using 3σ.

In order to demonstrate the analytical capacity of the proposed biosensor, a comparison of linear range and low detection limit with other non-enzyme based glucose sensors was listed in Table 1. Compared with some electrochemical sensors based on oxidation of glucose on metal or metal oxide modified electrodes

Table 1
Comparison of analytical performance of non-enzyme based glucose sensors.

Sensor	Linear range (μM)	Detection limit (μM)	References
Cu/CNTs/GCE	0.7–3500	0.2	Kang et al. (2007)
Cu_2O/MWCNT	0.05–5.0	0.1	Zhang et al. (2009)
Nanogold–plasmon-resonance–Con A	1000–40000	–	Aslan et al. (2004)
Fluorescein-labeled Con A–glycogen conjugate	5000–50000	–	Sato and Anzai (2006)
Con A/pDA/TH/GCE	1.0–100	0.75	Proposed

(Kang et al., 2007; Zhang et al., 2009), the biosensor based on Con A-glucose binding exhibited comparable performance, and it established a new route for glucose detection without direct oxidation of glucose-self. Compared with the sensors based on the same principle of Con A-glucose binding but with different detection methods, such as surface plasmon resonance and fluorescence (Aslan et al., 2004; Sato and Anzai, 2006), the proposed biosensor definitely has a relatively poor performance, however, it was an attempt by using the Con A-glucose binding for glucose detection via amperometric protocol. Although the proposed biosensor possessed a linear range which is not in the realm of blood sugar level, it can be used in real sample analysis just by diluting the sample.

3.5. Interferences

One of the most important analytical factors for a glucose biosensor is the ability to discriminate the interfering species from the target analyte. AA and UA presented in physiological fluids are the most important interferences for analysis of glucose, especially for non-enzymatic biosensors (Yuan et al., 2005). Herein, we studied the interference effect of 1.0×10^{-4} M UA and 1.0×10^{-4} M AA on the response of 4.0×10^{-5} M glucose. Experimental results showed that AA and UA did not give any observed interference to 4.0×10^{-5} M glucose of the biosensor (data not shown for brief), indicating that the biosensor possesses acceptable anti-interferent ability and excellent selectivity. As the principle for determination of D-glucose is based on the lectin–sugar binding, so it is reasonable to obtain the above results. It has to be mentioned that Con A also has different binding affinities for other sugar molecules, especially D-mannose, D-maltose, methyl-α-glucopyranoside, which can interfere with glucose detection. However, the interference effect on the measurement for glucose can be negligible due to their low concentrations under physiological conditions (Labib et al., 2010).

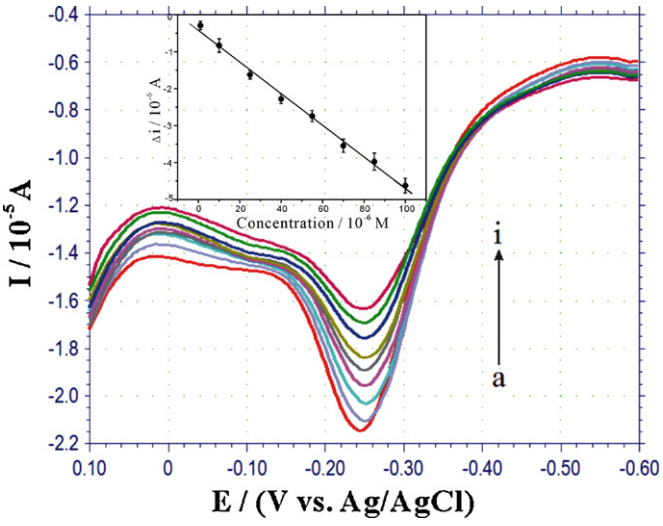

Fig. 5. DPV responses of Con A/pDA/Th/GCE to different concentrations of D-glucose: (a) 0, (b) 1.0×10^{-6} M, (c) 1.0×10^{-5} M, (d) 2.5×10^{-5} M, (e) 4.0×10^{-5} M, (f) 5.5×10^{-5} M, (g) 7.0×10^{-5} M, (h) 8.5×10^{-5} M, and (i) 1.0×10^{-4} M in 0.1 M PBS (pH 7.0). Inset shows the linear relationship between the difference of peak current and the concentration of D-glucose.

3.6. Reproducibility and stability of the biosensor

The reproducibility and stability are two important parameters for the evaluation of the performance of biosensors. The repeatability and stability of the proposed biosensor were examined by measuring the current response to 4.0×10^{-6} M D-glucose. The proposed biosensor showed an acceptable repeatability with a relative standard deviation (R.S.D.) of 3.1% for 11 successive assays by regeneration the Con A/TH modified GCE with 1.0×10^{-3} M dextran (39 kDa) after the biosensor binds of D-glucose. Six biosensors prepared independently using the same procedure gave a R.S.D. of 3.8%, indicating the biosensor has good reducibility. The stability of the Con A/pDA/TH/GCE was evaluated by intermittently measurement of 4.0×10^{-6} M D-glucose every 3 days, and it retained 91% of its initial response after 2 months storage. It is probable that the covalent immobilization of TH preserved TH suffering from loss. The exterior pDA film also has stabilization effect on TH layer and Con A, which dramatically enhanced the stability of the sensing platform.

3.7. Glucose determination in serum samples

This glucose biosensor could be applied to assay the real blood sugar. Three fresh serum samples were supplied by the Affiliated Hospital of Qingdao University and the samples were 200-fold diluted with 0.1 M PBS at pH 7.0 before analysis with the biosensor. By using standard addition method, 4.0×10^{-5} M D-glucose was added into the diluted samples, and measured current was converted to the glucose concentration based on the working curve. The recovery for the determination of glucose was in the range of 97.8–104.3% for three serum samples, indicating the proposed biosensor can be applied in real sample determination.

4. Conclusions

Selective determination of D-glucose was realized by a non-enzyme glucose amperometric biosensor based on Con A–glucose binding with TH as the response indicator. Due to the specific adherence mechanism of pDA through Michael addition and/or Schiff-base reaction with the amino group of TH and Con A, the sensing platform exhibited high stability, which was demonstrated by the fact that the signal of TH hardly decreased during multi-scannings and the good long-term stability of the biosensor. The specific lectin–sugar binding also endowed the biosensor with excellent anti-interference ability, and the biosensor showed acceptable recoveries in real serum sample determination. The non-enzyme glucose amperometric biosensor holds great potential in clinic blood sugar detection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.10.040.

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