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Microbial surface display of glucose dehydrogenase for amperometric glucose biosensor

Bo Liang [a,1], Liang Li [a,b,1], XiangJiang Tang [a], Qiaolin Lang [a], Hongwei Wang [a], Feng Li [b], Jianguo Shi [c], Wei Shen [d], Ilaria Palchetti [e], Marco Mascini [e], Aihua Liu [a,*]

[a] Laboratory for Biosensing, Qingdao Institute of Bioenergy & Bioprocess Technology, and Key Laboratory of Bioenergy, Chinese Academy of Sciences, 189 Songling Road, Qingdao 266101, China
[b] College of Environment and Safety Engineering, Qingdao University of Science and Technology, 53 Zhengzhou Road, Qingdao 266042, China
[c] Key Laboratory for Biosensors of Shangdong Province, Biology Institute of Shandong Academy of Sciences, 19 Keyuan Road, Jinan 250014, China
[d] Department of Chemical Engineering, Monash University, Clayton Campus, Wellington Rd, Vic 3800, Australia
[e] Dipartimento di Chimica, Universita degli Studi di Firenze, Via della Lastruccia 3, 50019 Sesto Fiorentino, Italy

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ABSTRACT

A genetically engineered *Escherichia coli* (*E. coli*) strain displaying glucose dehydrogenase (GDH) with ice-nucleation protein (INP) as the anchoring motif was first constructed. The surface localization and functionality of the fusion protein containing GDH were verified by SDS-PAGE, Western blotting and enzymatic activity assay. The fusion of INP had no effects on the functionality of GDH cofactor binding domain. The activity assay showed that 74.6% of the cell lysate GDH activity was detected in the outer membrane fractions. Compared with the crude enzyme solution from *E. coli* expressing intracellular GDH, the GDH-displaying bacteria (GDH-bacteria) was stable within pH 6–10 below 40 °C. Further, a novel electrochemical glucose biosensor was developed by construction of Nafion/GDH-bacteria/multiwalled-carbon-nanotube modified electrode. The as-prepared biosensor is linear with the concentration of D-glucose within the range of 50–800 μM and a low detection limit of 4 μM D-glucose (S/N=3). Excess saccharides including D-galactose, D-fructose, D-cellbiose, L-arabinose and D-sucrose, D-maltose, D-mannose and D-xylose as well as common interfering substances (acetaminophen, ascorbic acid and uric acid) did not affect the detection of D-glucose (0.1 mM). The proposed biosensor is stable, specific, reproducible, simple, rapid and cost-effective, which can be used for detection of real samples. It is envisioned that this GDH-bacteria will be found promising applications in biofuel cell, glucose detection and cofactor reproduction system.

1. Introduction

Glucose dehydrogenase (GDH) (EC 1.1.1.47) is the key enzyme in the course of spore producing stage in *Bacillus* sp (Sano et al., 1988). GDH originating from *Bacillus subtillus* was first isolated by Ramaley(Ramaley and Vasantha, 1983). The gene encoding this enzyme was cloned and expressed in *Escherichia coli* (*E. coli*) (Vasantha et al., 1983). GDH catalyzes the transformation of D-glucose to gluconolactone in the presence of coenzyme $NAD(P)^+$(nicotinamide adenine dinucleotide phosphate), which is reduced into NAD(P)H (the reduced form of nicotinamide adenine dinucleotide phosphate). As early as in the late 1980s, researches have been conducted on GDH, but the recombinant GDH showed low enzyme activity. Further, the high costs of purification and decline of enzyme stability limit its practical application.

Foreign proteins could be displayed on the surface of microorganisms such as phage, bacteria, yeast and other microbial cells by means of genetic engineering (Knowles et al., 2009). Microbial surface display would provide an economical alternative to the traditional production of enzyme because of the absence of purification, high activity and stability (Chen et al., 2011). To date, microbial surface display has been widely used in protein engineering, vaccine design, disease diagnosis, and environmental protection (van Bloois et al., 2011) and biological synthesis (Richter et al., 2010), however, it is rarely reported on bacteria-surface displayed enzyme for biosensing (Li et al., 2012, 2013) and biofuel cells (Fishilevich et al., 2009; Xia et al., in press) application. Many surface display systems have been developed so far. Among them, ice-nucleation protein (INP) has been widely used for surface display of foreign proteins (Khodi et al., 2012; Li et al.,

* Corresponding author. Tel.: +86 532 8066 2758.
E-mail address: liuah@qibebt.ac.cn (A. Liu).
[1] These two authors contributed equally to this work.

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2004, 2009; Wu et al., 2009; Yang et al., 2012). The structure of the membrane should be kept intact and host cells should maintain normal growth when foreign proteins displayed on the surface of cells. In this respect, INP can be used to display many proteins on the surface of various gram-negative bacteria without affecting cell viability (Jahns and Rehm, 2012; van Bloois et al., 2011). Nevertheless, issues are unclear that the carrier protein affects enzyme activity site and that the cofactor binding site of the passenger affects the activity of the cell displayed enzyme. In our previous work, surface display of xylose dehydrogenase using inaPb from *Pseudomonas borealis* was established (Liang et al., 2012), which showed the N-terminal domain of inaPb serving as an ideal anchoring motif for bacterial surface display system.

As we know, enzyme-based biosensors especially oxidoreductases have been mostly used as a biological recognition elements (Cavrois et al., 2002; Mikkelsen and Rechnitz, 1989). Among the oxidoreductases, glucose oxidase (GOD) is the most commonly used enzyme (Foulds and Lowe, 1988; Malitesta et al., 1990). GOD can selectively catalyze the oxidation of glucose to gluconolactone in the presence of oxygen (Zhu et al., 2007). However, the response of these biosensors is susceptible to oxygen concentration in the measuring media (Tang et al., 2001). Moreover, many other electroactive species commonly present in biological fluids can also be oxidized and cause false current response (Kohma et al., 2007; Shan et al., 2007). Amperometric biosensors based on glucose dehydrogenase (GDH) can be employed to overcome this problem (Scheltergraf et al., 1984; Wang et al., 2012; Yu et al., 2011; Zafar et al., 2012a). To date, pyrroloquinoline quinine dependent glucose dehydrogenase (PQQ-GDH) (Flexer et al., 2011; Schubart et al., 2012), flavin adenine dinucleotide(FAD)-dependent glucose dehydrogenase (FAD-GDH) (Tsujimura et al., 2006; Zafar et al., 2012a, 2012b) and NAD-dependent glucose dehydrogenase(NAD-GDH) (Appelqvist et al., 1985; Schelter-Graph et al., 1984) based amperometric biosensors have been developed. Among them, PQQ-GDH has low specificity and can oxidize a variety of saccharides such as mannose, maltose, lactose, etc., thereby causing interferences (Flexer et al., 2011; Igarashi et al., 2004). FAD-GDH based biosensors, on the other hand, experienced drawbacks such as complicated technique, high-cost, and difficulty in operation (Monošík et al., 2012; Tsujimura et al., 2006). Usually, NAD-GDH exhibits higher substrate specificity and stability than PQQ-GDH does (Cass et al., 1984; Jonsson et al., 1989; Lange and Chamber, 1985; Miki et al., 1989). Therefore, glucose can be determined selectively by detecting the anodic current of enzymatically liberated NADH (the reduced form of nicotinamide adenine dinucleotide).

Taken together, GDH-displaying bacteria (GDH-bacteria) will hold great promise in the development of electrochemical glucose biosensor with good sensitivity and selectivity and operational stability. In this paper, we demonstrate for the first time the functional cell-surface display of GDH with inaPb. Furthermore, we found that the impact of the N-terminal domain of inaPb on the NAD(P)$^+$ binding domain of GDH when the carrier protein anchors the passenger protein to the cell surface. And finally, the use of the GDH-bacteria for sensitive amperometric glucose biosensor is presented.

2. Materials and methods

2.1. Chemicals and reagents

Nafion (perfluorinated ion-exchange resin, 5 wt% solution in a mixture of lower aliphatic alcohols and water) was purchased from Aldrich and used as received. 0.05 wt% Nafion solution was prepared from 5 wt% Nafion. NAD$^+$(the oxidized form of nicotinamide adenine dinucleotide) was purchased from Bio Basic Canada Inc. Phosphate buffer saline (PBS, 0.1 M, pH 7.4) was used as the supporting electrolyte solution. All solutions were mutarotated overnight at room temperature before use. All other chemicals and reagents are of analytical grade and were prepared using ultrapure water.

2.2. Bacterial strains, plasmids, and culture conditions

E. coli BL21 (DE3) (F$^-$ *omp*T *hsd*S$_B$($r_B^- m_B^-$) *gal dcm*(DE3)) were used as the host for the surface display. The plasmid pET-*gdh* containing the *gdh* gene was kindly gifted by Dr. Xian Xu (College of Life Science and Pharmaceutical Engineering, Nanjing University of Technology, China) and used as a template for further PCR manipulations. The plasmid pTInaPb-N was used for construction and expression of fusion protein (Liang et al., 2012). *E. coli* cells carrying expression vectors were grown in Luria-Bertani (LB) medium supplemented with kanamycin (30 μg/mL) at 200 rpm and 37 °C. When the culture reached an OD_{600} value of 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM) was added to the culture for induction of recombinant protein expression, and then further cultivated overnight at 25 °C. The procedure for the cell fractionation reported in previous study was used with modification (Li et al., 2004; Liang et al., 2012).

2.3. SDS-PAGE and Western Blot analysis

Subcellular fractionated samples (cytoplasm, outer membrane and inner membrane) were analyzed by 12% (wt/vol) SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Then the separated proteins were electroblotted onto PVDF membrane. Mouse anti-His monoclonal antibody (Tiangen, China) was used as the primary antibodies. The secondary antibody was alkaline phosphatase conjugated anti-mouse IgG. Bound antibodies were detected with BCIP/NBT (Tiangen, China).

2.4. GDH activity assay

The whole cell GDH activity was measured by monitoring the production of NADH (the reduced form of nicotinamide adenine dinucleotide) at 340 nm. After 24 h culture upon IPTG induction, cells were harvested and diluted to unit cell density (OD_{600}=1.0) with 75 mM Tris–HCl buffer (pH 8). The standard assay mixture contained 5 mM D-glucose, 2 mM NAD$^+$, 100 μL of cells (OD_{600}=1.0) and 75 mM Tris–HCl buffer (pH 8). Activity was expressed in units (1 μmol NADH generated per min) per OD_{600} whole cells at 25 °C.

2.5. Effect of temperature and pH on the stability of cell displayed GDH

To test thermostability, cells were incubated in 75 mM Tris–HCl buffer (pH 8) at different temperatures from 20 °C to 60 °C for 1 h. The enzyme stability towards pH was investigated by incubating cells at 25 °C in buffer (pH 4–11) for 1 h. The residual activity was then measured under above assay condition.

2.6. Apparatus and measurements

SDS-PAGE and electroblotting were performed using Biorad Mini-PROTEAN Tetra Electrophoresis System and Biorad Mini Trans-Blot Cell (Bio-Rad Laboratories, Inc., USA), respectively. The UV-visible measurements were carried out using DU 800 UV/Vis Spectrophotometer (Beckman Coulter, Inc., USA) at room temperature. The electrochemical experiments were performed with a CHI660D electrochemical workstation (CHI, Chenhua, Shanghai, China). All experiments were carried out with a three-electrode system with a chemically modified glassy carbon electrode (GCE, 3 mm in diameter) as the working electrode, a

platinum wire as the auxiliary electrode, and a saturated calomel electrode (SCE) as the reference electrode.

2.7. Preparation of Nafion/GDH-bacteria/MWNTs composite film modified GCE

The GCE was successively polished to a mirror finish using 1.0, 0.3 and 0.05 μm alumina slurry followed by rinsing thoroughly with deionized water. After successive sonication in anhydrous ethanol and ultrapure water, the electrode was rinsed with ultrapure water and allowed to dry at room temperature. Multi-walled carbon nanotubes (MWNTs, 2 mg) were dispersed in 1 mL 0.05 wt% nafion solution with ultrasonication. MWNTs suspension (10 μL) was dropped on the surface of GCE and dried in air. Next, 10 μL of GDH-bacteria aqueous dispersion was dropped on the inverted GCE and dried overnight at 4 °C in refrigerator. At last, 10 μL of Nafion solution (0.05 wt%) was syringed to the electrode surface. Finally, the modified GCE was immersed in PBS to remove the loosely adsorbed bacteria and was stored at 4 °C in a refrigerator under dry conditions when not in use.

3. Results and discussions

3.1. Construction of GDH surface display system

The recombinant plasmid pTInaPbN-Gdh constructed in this study is shown in Fig. S1(Supplementry material). The hybrid of InaPb-N/Gdh was controlled under the T7 promoter operator. In the present work, *gdh* gene was fused into the same anchoring motif, and the expression of InaPb-N/Gdh was induced with IPTG at 25 °C for 24 h.

3.2. Surface localization of InaPb-N/GDH fusion in cell

Expression of the recombinant protein in *E. coli* was analyzed by SDS-PAGE and Western blot analysis (Fig. 1). Fusion proteins were probed by immunoblotting with Anti-His antibody, and this mature-size InaPb-N/Gdh (~51 kD) was detected in the outer membrane fraction. Target proteins were not detected in any fractions of the negative controls. Therefore, the InaPb-N/Gdh fusion was successfully displayed on the cell surface.

3.3. The GDH activity of recombination strain

GDH can convert D-glucose to D-xylonolactone with NAD^+ as its cofactor. The GDH activity can be detected with the production of NADH, which shows typical absorption peak at 340 nm. The GDH activity of whole cells and subcellular fraction samples was summarized in Fig. S2 (Supplementry material). The majority of GDH activity was detected in the outer membrane fraction (74.6% of the cell lysate activity). In contrast, very little activity was detected in the outer membrane fraction of control. These results suggested the correct translocation of fusion protein on the outer membrane of host cells and efficient surface display of GDH on *E. coli*. To the best of authors' knowledge, this is the first report on the functional display of GDH on bacterial cell surface.

In the current study, the INP motif was fused with the N terminal of GDH, which had been reported to harbor the $NAD(P)^+$ binding domain(Yamamoto et al., 2001). We expressed two GDH from the strain of *B. subtillus* L1 or from the plasmid pET-*gdh*, respectively. In contrast with using the plasmid pET-*gdh*, the GDH displayed from strain L1 actually exhibited no enzyme activity (data not shown), probably originating from the difference of three amino acid residues near the N terminal region of GDH between strain L1 (A45, Q46 and T47) and plasmid pET-*gdh* (P45, N46 and E47). All of the results further indicated that INP would not affect the $NAD(P)^+$ binding site of GDH and could serve as an ideal anchoring motif for bacterial surface display system.

3.4. Effect of temperature and pH on the stability of cell displayed GDH

For recombinant GDH, no obvious inactivation could be detected during incubation below 40 °C (Fig. 2A). The enzyme was able to retain ~70% of its activity for 60 min at 45 °C. However, the crude enzyme solution from *E. coli* expressing intracellular GDH, only ~35% of its activity was kept under the same conditions. The thermostability of the recombinant GDH observed here is in accordance with the results of previous studies in which the stability of foreign proteins was improved because of INP display system (Li et al., 2004; Yang et al., 2010). The *E. coli*-displaying GDH was most stable within pH 6–10. Enzyme activity was lost abruptly when the pH was lower than 6 or higher than 10 (Fig. 2B).

3.5. Electrocatalytic characterization of the Nafion/GDH-bacteria/MWNTs/GCE for glucose oxidation

Electrocatalytic oxidation of glucose at bare GCE was clearly observed in D-glucose solution containing GDH-bacteria and NAD^+(Supplementary material, Fig. S3), suggesting the excellent

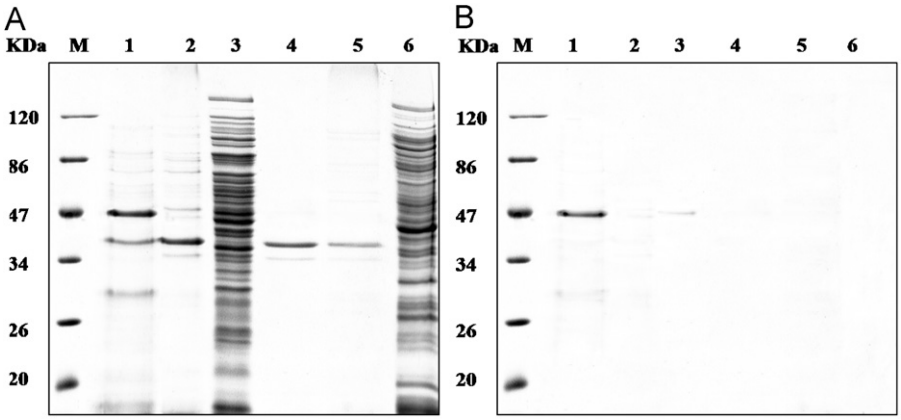

Fig. 1. (A) SDS-PAGE and (B) Western blot analysis of subcellular fractions. Lane M, protein standard markers; lane 1, outer membrane fraction of *E. coli* expressing vectors pTInaPb-N/Xdh; lane 2, inner membrane fraction of *E. coli* expressing vectors pTInaPb-N/Xdh; lane 3, cytoplasmic fraction of *E. coli* expressing vectors pTInaPb-N/Xdh; lane 4, outer membrane fraction of *E. coli* expressing pET28a (+); lane 5, inner membrane fraction of *E. coli* expressing pET28a (+); and lane 6, cytoplasmic fraction of *E. coli* expressing pET28a (+).

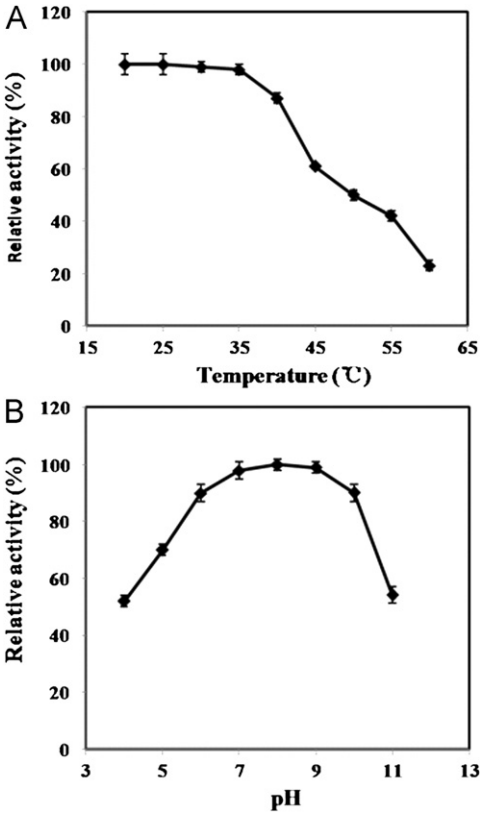

Fig. 2. Effects of temperature (A) and pH (B) on the stability of cell displayed GDH.

catalytic role of the cultured GDH-bacteria. Then, the GDH-bacteria were immobilized on the electrode surface incorporating with multiwalled carbon nanotubes. The GDH-bacteria loading was optimized in the cast film on the electrode surface in order to obtain the high sensitivity of the microbial biosensor (data not shown). It was found that 10 μL of GDH-bacteria loading showed the maximal oxidation current at 0.5 V. Therefore, 10 μL of GDH-bacteria solution was used for electrode modification throughout. The different modified electrodes were electrochemically characterized by linear sweep voltammetry (Fig. 3). Judging from the linear sweep voltammogram (LSV) of the Nafion/MWNTs/GCE (Fig. 3, curve a) in 0.1 M PBS (pH 7.4) containing 1 mM NAD^+ and 1 mM glucose, oxidation of glucose did not occur. Then, the Nafion/GDH-bacteria/MWNTs/GCE was also characterized by linear sweep voltammetry in 0.1 M PBS (pH 7.4) (Fig. 3, curve b) containing 1 mM NAD^+(Fig. 3, curve c), 1 mM glucose(curve d), 1 mM NAD^+ and 1 mM glucose (Fig. 3, curve e). In the presence of 1 mM NAD^+ and 1 mM glucose, a large oxidation current was observed with a very low onset potential of +0.2 V (vs. SCE) (Fig. 3, curve e). These results clearly demonstrate that the electrocatalytic oxidation of NADH to NAD^+ occurs directly at the MWNTs surface. As expected, GDH-bacteria functioned well as a dehydrogenase enzyme for glucose. Consequently, the observed excellent electrocatalysis of the Nafion/GDH-bacteria/MWNTs/GCE for the oxidation of glucose makes it to design a glucose biosensor.

3.6. Amperometric determination of glucose

The typical steady-state catalytic current–time response was obtained with Nafion/GDH-bacteria/MWNTs/GCE by using amperometry at an applied potential of +0.5 V (Fig. 4). The oxidation current increased after successive injection of 50 μM glucose and reached at 95% steady-state value within 1–2 s

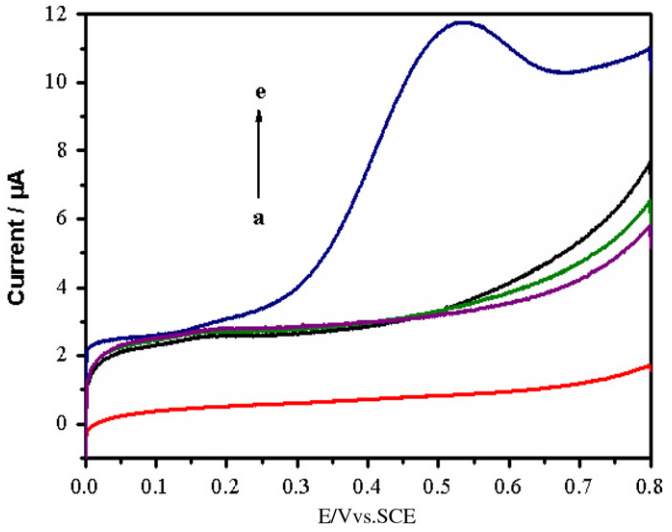

Fig. 3. LSVs of Nafion/GCE in 0.1 M PBS (pH 7.4) containing 1 mM NAD^+ and 1 mM glucose (a) and Nafion/GDH-bacteria/MWNTs/GCE in 0.1 M PBS (pH 7.4) (b), 1 mM NAD^+(c), 1 mM glucose (d) and 1 mM NAD^+ and 1 mM glucose (e). Scan rate, 50 mVs^{-1}.

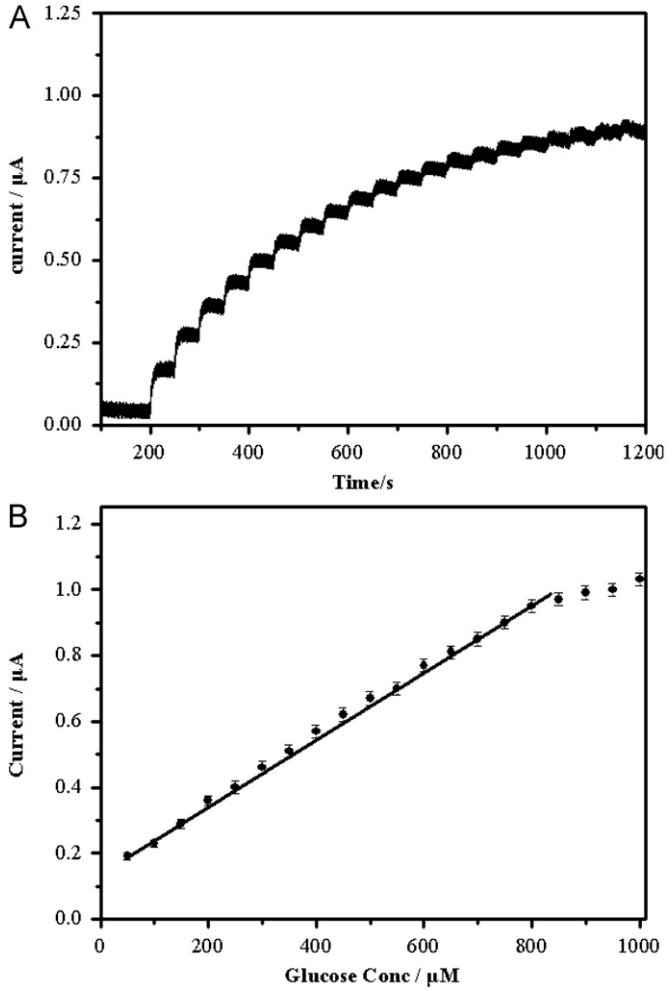

Fig. 4. (A) Current–time response of the Nafion/GDH-bacteria/MWNTs/GCE for the successive addition of 50 μM glucose at 0.5 V(vs. SCE) in 0.1 M PBS (pH 7.4) containing 1 mM NAD^+. (B) Typical calibration graph of the biosensor for glucose.

(Fig. 4A). Such a fast amperometric response is indicative of a fast overall oxidation of NADH in the enzymatic reaction at the modified electrode. The current response was linear with glucose concentration within 50–800 μM (Fig. 4B). The linear regression equation is $y=0.13924+0.00103x$ with a correlation coefficient of 0.999. The limit of detection (LOD) is 4 μM glucose (S/N=3). This dynamic range is much broader than those obtained using GOx/Ag/CNTs/chitosan film- (0.5–50 μM) (Lin et al., 2009), GDH/PPS/SWNT film- (50–700 μM) (Saleh et al., 2012) and GDH/CNTs/chitosan-glutaricdialdehyde film-modified electrode (5–300 μM) (Zhang et al., 2004). The limit of detection is much lower compared with those values of other typical GDH-based glucose biosensors reported previously (Saleh et al., 2012). Therefore, the excellent sensitivity of the proposed sensor is probably originating from the good assembly of the GDH on the bacteria surface. Actually, the display of enzymes can genetically control the enzyme proportion and the distance between enzymes on cell surface to facilitate the enzymatic reaction, which is unexpected for conventional enzyme immobilization.

3.7. Selectivity of the biosensor

The substrate (saccharides) sensitivity of the GDH-bacteria was further investigated. The current responses of this sensor to the successive addition of D-glucose (0.1 mM) and a series of other saccharides are shown in Fig. 5. Compared with the current for 0.1 mM D-glucose (Fig. 5, arrows a,b,l), D-galactose, D-fructose, D-cellbiose, L-arabinose and D-sucrose (each 10 mM) actually showed background currents (Fig. 5, arrows c–g), suggesting that there was no interference to the detection of 0.1 mM D-glucose in the presence of 100-fold excess of these substrates. In the case of D-maltose, D-mannose, D-xylose (each 0.5 mM) as substrates, very small oxidation current (about 10% current signal for 0.1 mM D-glucose) was observed due to the catalysis of the cell-displaying GDH, which meant fivefold excess of these saccharides would have a little interference on the detection of D-glucose. However, when the concentration of these saccharides was lowered (D-maltose, D-mannose and D-xylose, each 0.2 mM), oxidation current was undetectable (Fig. 5, arrows h–j), which indicated that two-times higher concentration of D-maltose, D-mannose and D-xylose will not affect the detection of D-glucose. It should be mentioned here that, compared with the serious interferences from D-maltose, D-mannose, etc for the PQQ-GDH based glucose biosensor (Flexer et al., 2011), the GDH-bacteria-based biosensor exhibited favorably selectivity to these substrates. Usually the concentration of D-glucose is much higher than that of these three saccharides in the actual degradation products of lignocelluloses and food samples (Brown, 1983), which suggests no interference from D-maltose, D-mannose, and D-xylose to the detection of D-glucose in real samples. Common interfering substances such as ascorbic acid, acetaminophen and uric acid (each 10 mM) did not affect the detection of D-glucose (0.1 mM) (Supplementary material, Fig. S4). Therefore, it is clear that this sensor can be used to selectively detect D-glucose without interference.

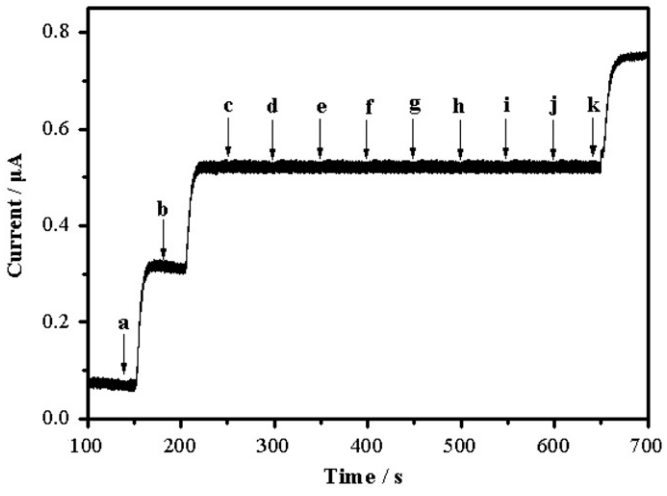

Fig. 5. Current–time curve obtained for the Nafion/GDH-bacteria/MWNTs/GCE on the successive addition of 0.1 mM D-glucose (a,b,k), 10 mM D-galactose (c), 10 mM D-fructose (d), 10 mM D-cellbiose (e), 10 mM L-arabinose (f), 10 mM D-sucrose (g), 0.2 mM D-maltose (h), 0.2 mM D-mannose (i), 0.2 mM D-xylose(j) in 0.1 M PBS buffer (pH 7.4) containing 1 mM NAD^+.

3.8. Operational stability of the biosensor

The present biosensor was carried out by the continuous measurement of 0.1 mM glucose at the applied potential of 0.5 V over a period of more than 25 min (Supplementary material, Fig. S5). An amperometric response with less than 10% signal drift demonstrated a considerable resistance against the so-called electrode fouling, which could be attributed to its three-dimensional network. In addition, a large surface area of the MWNTs allows a large amount of GDH to be immobilized, resulting in a MWNTs-based enzyme reservoir.

3.9. Analysis of real samples

The Nafion/GDH-bacteria/MWNTs/GCE bioelectrode was used to detect D-glucose in real samples including peach juice and products of straw degradation products. The samples were accurately diluted with buffer solution to fit into the linear range of the sensor before measurement. For each sample, three determinations were performed. The concentrations were calculated based on the calibration graph and are shown in Table S1 (Supplementry material). The RSD values are within 9%, demonstrating that the precision and stability of the calibration graph are suitable for quantifying D-glucose in real samples. The recoveries of the added D-glucose standards are within 97.3–102.7%, which reveal good accuracy of the proposed method.

4. Conclusions

In summary, GDH displaying bacteria system was successfully constructed for the first time using inaPb from *Pseudomonas borealis*. The fusion protein was about 51 kD, and the majority of GDH activity was detected in the outer membrane fraction. INP would not affect the $NAD(P)^+$ binding site of GDH. The cell displayed GDH was most stable within pH 6–10 below 40 °C. Furthermore, we developed a novel Nafion/GDH-bacteria/MWNTs nanocomposite electrode. The modified electrode is rapid in response, sensitive and broad in detection linear range (50–800 μM). Additionally, the proposed microbial biosensor exhibited good reproducibility and good operational stability as well as non-interference from other saccharides and other common interfering agents. Considering its excellent properties, it is anticipated that this GDH-bacteria will be found important potential applications in biofuel cell, glucose detection and cofactor reproduction system.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.01.050.

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