Chapter 13

Electrochemical Glucose Biosensor Based on Glucose Oxidase Displayed on Yeast Surface

Hongwei Wang, Qiaolin Lang, Bo Liang, and Aihua Liu

Abstract

The conventional enzyme-based biosensor requires chemical or physical immobilization of purified enzymes on electrode surface, which often results in loss of enzyme activity and/or fractions immobilized over time. It is also costly. A major advantage of yeast surface display is that it enables the direct utilization of whole cell catalysts with eukaryote-produced proteins being displayed on the cell surface, providing an economic alternative to traditional production of purified enzymes. Herein, we describe the details of the display of glucose oxidase (GO_x) on yeast cell surface and its application in the development of electrochemical glucose sensor. In order to achieve a direct electrochemistry of GO_x, the entire cell catalyst (yeast-GOx) was immobilized together with multiwalled carbon nanotubes on the electrode, which allowed sensitive and selective glucose detection.

Key words Yeast surface display, Glucose oxidase, Electrochemical biosensor, Glucose

1 Introduction

Glucose-1-oxidase (GO_x) (beta-D-glucose:oxygen-1-oxidoreductase, EC 1.1.3.4) from *Aspergillus niger* is capable of oxidizing beta-D-glucose to D-gluconolactone and releasing hydrogen peroxide, with glucose as electron donor [1]. The virtue of its glucose specific oxidation and extreme stability compared with other enzymes has allowed GO_x to play the leading role in enzyme electrodes construction for easy-to-use blood sugar testing [2]. It has been estimated that the world's market value of biosensors reaches about 5 billion US dollars, of which 85 % is attributed to glucose biosensors [3]. However, purified GO_x is required for immobilization on electrode, increasing the cost. In addition, the conventional physical or chemical immobilization approaches often result in enzyme activity loss and reduction in fractions immobilized over time, and increase the difficulty of mass transfer, which has become the bottleneck in the area of enzyme based electrode development [4].

Bin Liu (ed.), *Yeast Surface Display: Methods, Protocols, and Applications*, Methods in Molecular Biology, vol. 1319,
DOI 10.1007/978-1-4939-2748-7_13,

In the last decade, microbial surface display allowed direct utilization of whole cell catalysts with recombinant protein displayed on the cell surface, providing a low cost alternative to the purified enzymes. Robust surface display systems on bacterial (e.g., *E. coli*, *B. anthracis*) and yeast (e.g., *S. cerevisiae* and *Y. lipolytica*) had been developed and applied in peptide library screening, antibody production, live vaccine development and whole-cell biocatalysts construction, while the application in biosensor design is rare [5]. Recently, efficient *E. coli* surface display of xylose dehydrogenase [6] and glucose dehydrogenase [7, 8] has been developed. Meanwhile, sensitive and selective D-xylose biosensor [9, 10], D-glucose biosensor [7, 8] and assembly of xylose-based biofuel cell [11] were prepared by using *E. coli* surface displayed system. However, this prokaryotic system shows a major limitation as those large and complex proteins derived from eukaryotes often do not fold properly in *E. coli*. Therefore, it is necessary to develop an eukaryotic cell surface display system for biosensor design. The active GO_x with improved pH and thermal stability had been successfully expressed in *S. cerevisiae* [12]. We hereby describe in detail bioelectrode construction using the yeast displayed GO_x and carbon nanotubes. The exploration of this interesting whole-cell biocatalyst in the electrochemical glucose biosensing is presented.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature unless indicated otherwise. Carefully follow all waste disposal regulations when disposing waste materials.

2.1 GO_x Display on Surface of S. cerevisiae

1. GO_x deriving strain: *Aspergillus niger* CBS 513.88.
2. Yeast display system: pYD1 Yeast Display Vector Kit (Invitrogen, Carlsbad, CA).
3. YPD media: Dissolve 20 g peptone, 10 g yeast extract, and 15 g agar (optional) in 900 mL of water and autoclave for 20 min. Then, add 100 mL of 20 % filter-sterilized glucose.
4. Selection media: Dissolve 6.7 g YNB, 0.74 g Trp DO Supplement, and 15 g agar (optional) in 900 mL of water and autoclave for 20 min. Then, add 100 mL of 20 % filter-sterilized glucose.
5. Induction media: Dissolve 6.7 g YNB, 0.74 g Trp DO Supplement, and 15 g agar (optional) in 900 mL of water and autoclave for 20 min. Then, add 100 mL of 20 % filter-sterilized galactose.
6. Restriction enzymes (MBI Fermentas, Canada).

7. Yeastmaker™ Yeast Transformation System 2 (Clontech, Japan).
8. 0.1 M phosphate buffer saline (PBS) (pH 7.4): Dissolve 2.90 g disodium phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$), 0.24 g potassium phosphate monobasic (KH_2PO_4), 0.20 g potassium chloride (KCl), and 8.0 g sodium chloride (NaCl) with ultrapure water and dilute to 1,000 mL in volumetric flask. Adjust pH of the solution to 7.4 and autoclave.

2.2 Glucose Oxidase Activity Assay

1. Peroxidase (POD) solution: Immediately before use, dissolve 1 mg POD Type II (Sigma-Aldrich, USA) into 3 mL of cold water to make a solution containing about 60 Purpurogallin units/mL of POD. Store on ice.
2. 0.1 M PBS buffer (pH 7.4): Dissolve 2.90 g $Na_2HPO_4{\cdot}12H_2O$, 0.24 g KH_2PO_4, 0.20 g KCl, and 8.0 g NaCl with ultrapure water and dilute to 1 L in volumetric flask. Adjust pH of the solution to 7.4 and autoclave.
3. *o*-dianisidine solution: Dissolve 20 mg *o*-dianisidine dihydrochloride (Sigma-Aldrich, USA) in 8 mL water and then dilute 5.35–200 mL with 0. 1 M PB (0.21 mM *o*-dianisidine). Store at 4 °C protected from light.
4. Glucose solution: Dissolve 10 g (w/v) D-(+)-glucose (Sigma-Aldrich, USA) in 100 mL distilled water.
5. Reaction cocktail: Immediately before use, combine 192 mL *o*-dianisidine solution with 40 mL of glucose solution (0.17 mM *o*-dianisidine and 1.72 % glucose solution).
6. PB-10 pH meter (Sartorius AG, Germany).
7. UV/Vis Spectrophotometer (Beckman Coulter, Inc., USA).
8. Substrate solution for specific test: Dissolve 0.901 g D-(+)-glucose, 1.711 g D-(+)-maltose, 1.711 g D-sucrose, 0.751 g D-(+)-xylose, 0.901 g D-(−)-fructose, 0.751 g D-(−)-ribose, 0.901 g D-(+)-galactose, 0.901 g D-(+)-mannose, 0.751 g L-(+)-arabinose, and 1.711 g D-(+)-cellobiose in 100 mL distilled water to make a 50 mM substrate stock solution, respectively and store at 4 °C before use. All the sugars can be purchased from Sigma-Aldrich.
9. Cocktail for substrate test: Immediately before use, combine 19.2 mL *o*-dianisidine solution with 4 mL of glucose solution (10.69 mM substrate).

2.3 Preparation of Modified Electrode

1. 5 % Nafion (perfluorinated ion-exchange resin, 5 wt% solution in a mixture of lower aliphatic alcohols and water) (Aldrich Corporation, USA).
2. Multi-walled carbon nanotubes (MWNTs) (Wako Chemical Co., Tokyo, Japan).
3. 1-, 0.3-, and 0.05-μm alumina slurry (Aldrich Corporation) is dispersed with water under sonication before use.

4. MWNTs-Nafion suspension: 2 mg of MWNTs powder is dispersed in 1 mL of 0.05 wt% Nafion solution and ultrasonication for at least 10 min.
5. Glassy carbon electrode (GCE, 3 mm in diameter) (Chenhua Co., Shanghai, China).

2.4 Electrochemical Glucose Detection

1. 0.1 M PBS buffer (pH 7.4): Dissolve 2.90 g $Na_2HPO_4 \cdot 12H_2O$, 0.24 g KH_2PO_4, 0.20 g KCl, and 8.0 g NaCl with ultrapure water and dilute to 1,000 mL in volumetric flask. Adjust pH of the solution to 7.4.
2. 1.000 M glucose stock solution: Dissolve 900.8 mg β-D-(+) glucose in 5 mL 0.1 M PBS buffer (pH 7.4).
3. Yeast-GO_x, *see* Subheading 2.1.
4. 660D electrochemical workstation (CH Instruments, Chenhua Co., Shanghai, China).
5. Reference electrode: saturated calomel electrode (SCE, Chenhua Co., Shanghai, China).
6. Counter electrode: platinum wire (Chenhua Co., Shanghai, China).
7. 99.99 % N_2 gas.

3 Methods

3.1 Preparation of Yeast Catalyst

3.1.1 GO_x Displaying on Surface of *S. cerevisiae*

A frequently used gene encoding GO_x (GenBank accession No. J05242.1) may be amplified from genomic DNA of *A. niger* CBS 513.88 or its mutant stains. The displayed GO_x could be obtained by using pYD1 Yeast Display Vector Kit where the verified ORF is initially cloned into multiple cloning site (MCS) of pYD1 vector and transformed into *S. cerevisiae* EBY100. The details have been described in the manual of the kit, which used the a-agglutinin receptor of *S. cerevisiae* to display foreign proteins on the cell surface [13].

3.1.2 Expression Induction

1. Inoculate a single large yeast colony into 10 mL selection medium containing 2 % glucose and grow overnight at 30 °C with shaking at 200 rpm.
2. Read the absorbance of the cell culture at 600 nm (OD_{600}) until it reaches between 2 and 5.
3. Centrifuge the cell culture at 3,000–5,000 × *g* for 5–10 min and harvest the cell pellets (*see* **Note 1**).
4. Resuspend the cell pellet in tryptophan drop out medium containing 2 % galactose to an OD_{600} of 0.5–1 (*see* **Note 2**).
5. Induce the recombinant protein expression by incubating the cell culture at 20–22 °C with shaking at 200 rpm.

6. Remove a volume of cells equivalent to at least 1 OD_{600} units and assay the cell culture from 24 to 48 h time period (i.e., 24, 30, 36, 48 h) to obtain the cell culture with maximum display (*see* **Note 3**).
7. Harvest the cells by centrifuge at 3,000–5,000 × *g* for 5–10 min at room temperature. Resuspend the cell pellets with sterilized water by gentle shaking, centrifuge again and repeat this step. Finally, resuspend the yeast cells in 0.01 M PBS with a concentration of at least 1 OD_{600} per 10 μL, which could be stored at 4 °C as the cell stock for further analysis (*see* **Note 4**).

3.1.3 Glucose Oxidase Activity Assay

Glucose oxidase activity is determined by the coupled *o*-dianisidine peroxidase reaction [14]. Here, a modified protocol for the GO_x activity of whole cell catalyst is described.

1. Sequentially pipette 2.9 mL of reaction cocktail, 0.1 mL of POD, and 0.1 mL yeast-GO_x (PBS for blank) in suitable cuvettes.
2. Immediately mix by pipetting and record the increase in A_{500nm}/min for 10 min at 25 °C or room temperature. Obtain the maximum linear rate for both the test and the blank using a minimum of a 1 min period.
3. To determine the amount of displayed GO_x, add 0.1 mL yeast cells in 0.9 mL PBS and record the OD_{600}.
4. Calculate the GO_x activity (U).

$$U = 3.1 \times \left(\Delta A_{500nm} \,/\, \text{min test} - \Delta A_{500nm} \,/\, \text{min blank}\right) / \left(7.5\right) \times OD_{600nm}$$

where 7.5 is millimolar extinction coefficient of oxidized *o-dianisidine* at 500 nm [15]. One unit of GO_x activity is defined as the amount of enzyme required to oxidize 1 μmol of glucose/min under the above assay conditions (*see* **Note 5**).

3.1.4 Stability and Specificity Test of Yeast Displayed GO_x

1. To test the thermostability, incubate 0.1 mL of the yeast-GO_x at different temperatures from 20 °C to 60 °C for 1 h using a water bath and put the samples on ice until enzyme assay (*see* Subheading 3.1.3) (*see* **Note 6**).
2. To test the pH stability, resuspend 0.1 mL of yeast-GO_x stock in the same volume of test buffers (pH 4–11) and incubate at 25 °C for 1 h. Then, centrifuge the yeast-GO_x at 5,000 × *g* for 5 min and resuspend the pellets in 0.01 M PBS. Put samples on ice until enzyme assay (*see* Subheading 3.1.3) (*see* **Note 7**).
3. To test the substrate specificity, carry out a set of enzyme assay as described in Subheading 3.1.3 but use a substrate test cocktail, which includes 10 mM of different substrates such as D-GLUCOSE, D-maltose, D-sucrose, and D-xylose (*see* **Note 8**).

3.2 Preparation of the Modified Electrode

1. The bare GCE is polished carefully with 1.0-, 0.3-, and 0.05-μm alumina slurries, and then sonicated in anhydrous ethanol and distilled deionized water, respectively.
2. Rinse the electrode with ultrapure water and allow it to dry at room temperature.
3. Then, deposit 10 μL of MWNTs-Nafion suspension on the surface of GCE by dropwise pipetting and dry in air (*see* **Notes 9** and **10**).
4. After drying, 10 μL of GO_x-yeast aqueous dispersion is added dropwise on the inverted GCE and dried overnight at 4 °C in a refrigerator (*see* **Note 11**).
5. Before use, syringe 10 μL of Nafion solution (0.05 wt%) to the electrode surface and air-dry.
6. Finally, the modified GCE should be immersed in PBS to remove any loosely adsorbed GO_x-yeast and stored at 4 °C in a refrigerator under dry conditions. The thus-modified electrode is denoted as GCE/MWNTs/GO_x-yeast/Nafion.

3.3 Bio-nano Electrode for Glucose Sensing

3.3.1 Calibration Curve of Glucose

1. Prepare glucose standard solution with concentrations ranging from 0 to 14 mM with 0.1 M PBS buffer from 1.000 M glucose stock solution (*see* **Note 12**).
2. Measure cyclic voltammograms (CVs) at scan rate of 50 mV/s in glucose solution under the ambient-N_2 condition using a three-electrode system containing a GCE/MWNTs/GO_x-yeast/Nafion as the working electrode, a Pt wire as the auxiliary electrode, and a SCE as the reference electrode (Fig. 1a) (*see* **Notes 13** and **14**).
3. The cathodic peak currents (i_{pc}) at about −0.5 V from the CVs are measured. Further, the cathodic peak current change (Δi_{pc}) varying glucose concentration is calculated (*see* **Note 15**).
4. Make standard curve for glucose by plotting Δi_{pc} as a function of glucose concentration (Fig. 1b).

3.3.2 Real Sample Measurements

1. Measure CVs at scan rate of 50 mV/s in a 0.1 M PBS buffer solution under the ambient-N_2 condition. Carry out three repetitive measurements.
2. Measure CVs at scan rate of 50 mV/s in a 5.00 mL sample solution under the ambient-N_2 condition. Carry out three repetitive measurements. Calculate $\Delta i_{pc,sample}$.
3. Spike 5 μL of 1.00 M glucose standard solution into the above 5.00 mL sample solution and stir well. Perform CV measurements again. Carry out three repetitive measurements. Calculate $\Delta i_{pc,sample+std}$.

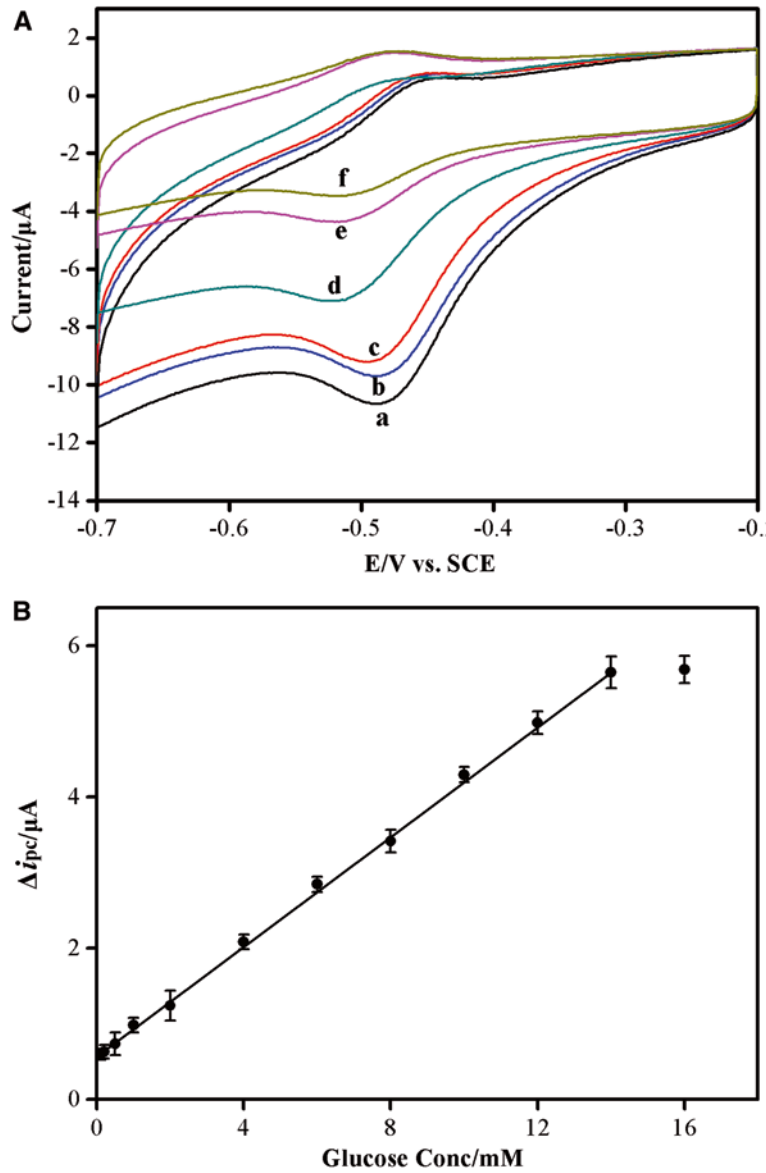

Fig. 1 (**A**) CVs of GCE/MWNTs/GO_x-yeast/Nafion in PBS buffer (pH 7.4) containing different concentrations of glucose: 0.0 mM (curve a), 0.1 mM (curve b), 0.5 mM (curve c), 2.0 mM (curve d), 8.0 mM (curve e), and 12.0 mM (curve f). Scan rate, 50 mV/s. (**B**) Typical calibration graph of the glucose biosensor. (Reprinted with permission from H. Wang et al., *Anal Chem* 2013, **85**, 6107–6112. Copyright©2014 American Chemical Society)

4. The glucose levels (C, mM) in the sample solution can be calculated as below:

$$C(\mathrm{mM}) = 5 \times \Delta i_{\mathrm{pc,sample}} \,/\, \left(\Delta i_{\mathrm{pc,sample+std}} - \Delta i_{\mathrm{pc,sample}}\right)$$

It may be necessary to dilute the sample solution with PBS buffer before measurement (*see* **Note 16**).

4 Notes

1. If possible, pipette out the residual medium attaching the tubes. The residual glucose may affect the expression induction of recombinant protein, as the Gal1 promoter is preferable to be regulated by glucose rather than galactose.
2. This is to ensure that the cells continue to grow in log-phase.
3. In our case, we found the 30–36 h often provided the optimal time point for maximum display with 0.15 U/OD (Fig. 2b).
4. The washing step will get rid of the residual protease in cell culture. It may be not necessary to bring the cell stock to an exact concentration, which could be determined in the further dilution steps. Generally, this cell stock could be stored 4 °C for less than 1 month without apparently affecting the enzyme activity or resulting in enzyme leakage.
5. The GO_x activity assay is generally sufficient. For further verification, proceed to staining of displayed proteins using an appropriate anti-tag antibody as described in the manual of pYD1 Yeast Display Vector Kit (Fig. 2a).
6. Usually, the GO_x from *A. niger* generally exhibited good activity below 50 °C. In our case, the immobilization on cell wall would improve thermostability of GO_x. Specifically, the displayed enzyme retained over 84.2 % of its activity at 56 °C, while 41 % enzyme activity maintained at 60 °C for 60 min. Besides, the intensive glycosylation of expressed protein by *S. cerevisiae* also affects enzyme thermostability.
7. Most of GO_x from *A. niger* is stable within pH 4–8. In our case, approximately 53 % of enzyme activity was lost when the pH was <3.0, whereas over 83 % of enzyme activity was maintained within pH 3.5–11.5.
8. 10 mM of substrate may be a proper starting point, as it still can provide excessive substrates in case of D-glucose. However, further substrate dilution steps (e.g., 5 mM, 1 mM, 0.1 mM) might be necessary for D-glucose, D-maltose, and D-sucrose, which structurally compose of glucose units. The difference of substrate specificity is supposed to be more obviously shown when a lower substrate concentration is used in enzyme assay. In our case, signal was almost nondetectable when other saccharides such as D-xylose, D-fructose, D-ribose, D-galactose, D-mannose, L-arabinose, and cellobiose were used as substrate. However, D-maltose and D-sucrose showed obvious interference at their high concentrations. Specifically, 5 mM (50-fold), 1 mM (10-fold), and 0.5 mM (5-fold) excess D-maltose had the signal accounted for 19.2 %, 2.6 %, 0.5 % of those values for 0.1 mM

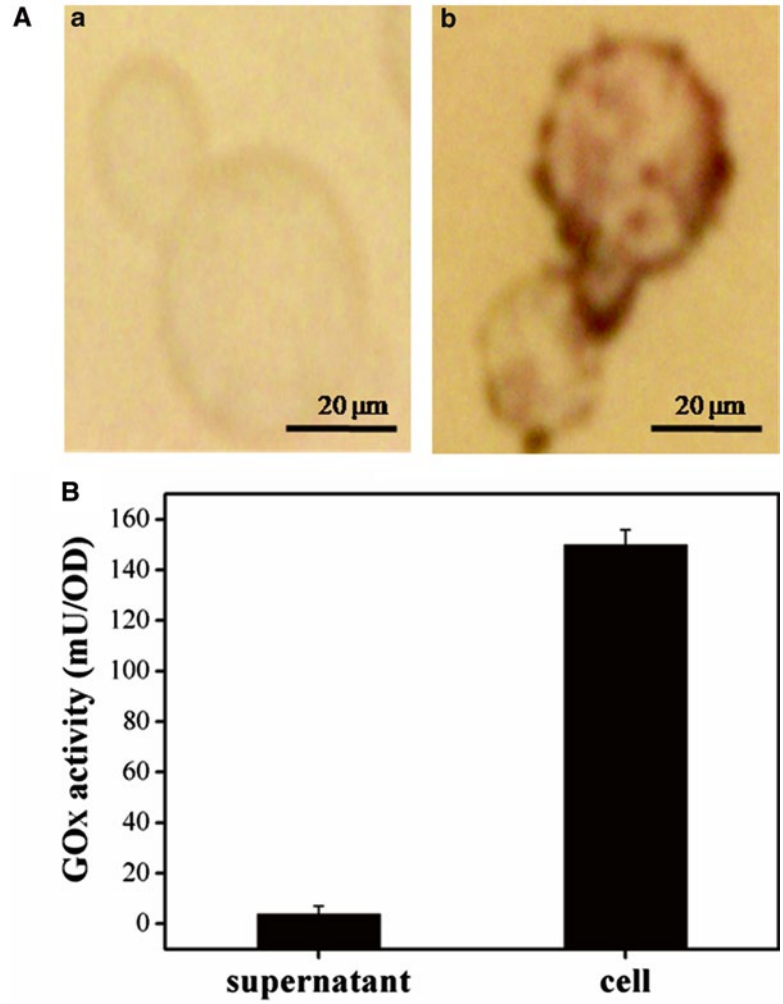

Fig. 2 (**A**) Pictures of (a) yeast cell control and (b) GO_x surface-displayed yeast. Cells was stained with anti-his antibody, followed by alkaline phosphatase staining. (**B**) GO_x activity of the surface-displayed yeast cells. (Reprinted with permission from H. Wang et al., *Anal Chem* 2013, **85**, 6107–6112. Copyright©2014 American Chemical Society)

glucose, separately. 10 mM (100-fold), 5 mM (50-fold), and 1 mM (10-fold) excess D-sucrose had the signal accounted for 15.3 %, 6 %, 0 % of those values for 0.1 mM glucose, separately.

9. 0.05 wt% Nafion was prepared from 5 wt% Nafion with absolute ethyl alcohol and used for carbon nanotubes dispersing and fixing agent.
10. It also makes sense to immerge the bare GCE into the MWNTs-Nafion suspension, however, by which the amount of MWNTs on the electrode surface is not controllable.

11. Before depositing the yeast-GO_x, the cell suspension should be diluted to about 1 OD_{600} per 100 μL with 0.1 M PBS. Excess loading of the yeast may lead to the cell leakage from electrode due to the ineffective crosslink during the immobilization process.
12. Standard curve can be prepared using a series of glucose standard solution.
13. The typical reaction mechanism is shown in Eqs. 1 and 2:

$$\text{Glucose} + \text{GO}_x\left(\text{FAD}\right) \rightarrow \text{Gluconolactone} + \text{GO}_x\left(\text{FADH}_2\right) \quad (1)$$

$$\text{GO}_x\left(\text{FADH}_2\right) + \text{O}_2 \rightarrow \text{GO}_x\left(\text{FAD}\right) + \text{H}_2\text{O}_2 \quad (2)$$

where FAD is flavin adenine dinucleotide, $FADH_2$ is the reduced form of FAD.

14. The Δi_{pc} is defined as the difference between the i_{pc} from CVs of the modified electrode in the presence of glucose and CV of the modified electrode in bare buffer solution.
15. A standard curve shows a linear range of 0.1–12 mM glucose.
16. To ensure experiment result exact, the samples should be necessary pretreated. Further, to filtrate through 0.22-μm membrane and dilute the sample solution with PBS buffer before measurement.

Acknowledgements

This work was supported in part by National Natural Science Foundation of China (91227116, 31200982, 31200598, 21275152, and 21475144), and the Hundred-Talent-Project (No. KSCX2-YW-BR-7), the Chinese Academy of Sciences.

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