Biosensors and Bioelectronics 51 (2014) 158–163

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Co-immobilization of glucoamylase and glucose oxidase for electrochemical sequential enzyme electrode for starch biosensor and biofuel cell

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ARTICLE INFO

Article history:
Received 19 April 2013
Received in revised form
9 July 2013
Accepted 10 July 2013
Available online 30 July 2013

Keywords:
Glucoamylase
Glucose oxidase
Sequential enzyme biosensor
Starch biosensor
Biofuel cell

ABSTRACT

A novel electrochemical sequential biosensor was constructed by co-immobilizing glucoamylase (GA) and glucose oxidase (GOD) on the multi-walled carbon nanotubes (MWNTs)-modified glassy carbon electrode (GCE) by chemical crosslinking method, where glutaraldehyde and bovine serum albumin was used as crosslinking and blocking agent, respectively. The proposed biosensor (GA/GOD/MWNTs/GCE) is capable of determining starch without using extra sensors such as Clark-type oxygen sensor or H_2O_2 sensor. The current linearly decreased with the increasing concentration of starch ranging from 0.005% to 0.7% (w/w) with the limit of detection of 0.003% (w/w) starch. The as-fabricated sequential biosensor can be applicable to the detection of the content of starch in real samples, which are in good accordance with traditional Fehling's titration. Finally, a stable starch/O_2 biofuel cell was assembled using the GA/GOD/MWNTs/GCE as bioanode and laccase/MWNTs/GCE as biocathode, which exhibited open circuit voltage of ca. 0.53 V and the maximum power density of 8.15 μW cm^{-2} at 0.31 V, comparable with the other glucose/O_2 based biofuel cells reported recently. Therefore, the proposed biosensor exhibited attractive features such as good stability in weak acidic buffer, good operational stability, wide linear range and capable of determination of starch in real samples as well as optimal bioanode for the biofuel cell.

1. Introduction

As one of the most general carbohydrates in crops, starch is usually used as food processing auxiliary to improve the taste and nutrition, and can also be used as filler of composite materials for the degradation of certain synthetic polymers due to its character of innocuity and easy degradation (Star et al., 2004). Due to its cost-effectivity, starch is also considered as a good fuel for biofuel cells. Desirable technological, organoleptic, and nutritional properties in the end products are all dependent on the addition of starch in the processes such as the baking of bread, the production of pasta products and starch-based snack foods, breakfast cereals, pregelatinized flour, baby foods, and parboiled cereals (Olkku and Rha, 1978; Lineback and Wongsrikasem, 1980; Lund and Lorenz, 1984). The level of starch content in food or pill is a vital parameter in quality inspection of brewing, food industry and pharmacy. Traditional ways to detect starch include polarimetric (Garcia and Wolf, 1972) and the Fehling titration method (Menyhert, 1908), however, they are complex and time-consuming in sample pretreatment. Especially, the results of Fehling titration method are greatly affected by the interference from other possible reduced sugars co-existed in the sample. On the other hand, the enzyme-based electrodes in combination with hydrogen peroxide sensors (Cordonnier et al., 1975; Mascini et al., 1983) or oxygen sensors (Coulet and Bertrand, 1979; Bardeletti and Coulet, 1987) were reported for starch measurement, which were based on the determination of the reduced saccharides, the hydrolytic products of starch, nevertheless, it is laborious. Sequential biosensors containing two or more enzymes which catalyze substrate in sequence, are mostly used in the determination of disaccharides (Zhang and Rechnitz, 1994; Zhang, 2000), starch (Abdul Hamid et al., 1990) and cholesterol (Motonaka and Faulkner, 1993). The performance of this kind of biosensors greatly depends on the amount, ratio, and distribution control of two enzymes as well as the immobilization methods (Zhou et al., 2001). However, the sensitivity and operational stability are usually not so satisfactory

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http://dx.doi.org/10.1016/j.bios.2013.07.021

compared with single enzyme biosensor, probably arising from the complexity in enzyme membrane preparation (Zhou et al., 2001). Electrochemical sequential electrode was also used in the surface-displaying enzyme microbial fuel cell (Bahartan et al., 2012).

Biofuel cell (BFC) which employs enzymes or/and microorganisms as the biocatalysts for the production of electricity from renewable organic matter, represents a new kind of green power sources and has attracted much attention in recent years (Bullen et al., 2006; Du et al., 2007; Cracknell et al., 2008). There are intensive studies on the design and characterization of enzyme-based BFCs, however, most of work has been focused on using monosaccharides such as glucose or xylose as fuels (Li et al., 2009; Gao et al., 2010; Wen et al., 2011; Zebda et al., 2011; Xia et al., 2013). In comparison with monosaccharides, starch represents an alternative energy source with lower cost and easier processing procedures. By far, starch has been used as energy resource in microbial fuel cells (Velasquez-Orta et al., 2011; Herrero-Hernandez et al., 2013). However, there are no reports on BFCs based on sequential enzyme bioelectrocatalysis of starch.

Glucoamylase (GA, α-1,4-glucan-gluco-hydrolase, EC. 3.2.1.3) is a starch hydrolyzing enzyme which catalyzes the hydrolysis of α-(1,4) glycosidic bonds at the non-reducing end of starch polymer to release free glucose (Marin-Navarro and Polaina, 2011). GA is an important enzyme extensively used in bio-industry for production of starch sugar, alcohol and single-cell protein (Velasquez-Orta et al., 2011; Yamakawa et al., 2012). As an essential polysaccharide hydrolase, GA is widely used in the hydrolysis of starch into glucose before their measurement with either Fehling's titration or electrochemical method (Abdul Hamid et al., 1990; Zhang and Rechnitz, 1994; Zhang, 2000).

In the present study, we constructed a sequential biosensor based on the co-immobilization of GA and glucose oxidase (GOD) for the determination of starch. With the incorporation of carbon nanotubes, which could facilitate the direct electron transfer between electrode and GOD, the redox center (flavin adenine dinucleotide, FAD) of GOD presented direct electrochemistry. The reduction peak current decreased with the increasing of glucose in solution based on the oxygen consumption (Wang et al., 2009). The proposed biosensor enabled to determine starch without the measurement of H_2O_2, thus simplified starch biosensor and biofuel cell. To the best of our knowledge, this is the first report on the construction of starch biosensor without extra sensors such as Clark-type oxygen sensor or H_2O_2 sensor. Finally, a starch/O_2 biofuel cell was assembled using the GA/GOD/MWNTs/GCE electrode as bioanode and laccase/MWNTs/GCE as biocathode, which exhibited open circuit voltage up to ca. 0.53 V and the maximum power density of 8.15 μW cm^{-2} at 0.31 V, comparable with the other glucose/O_2 based biofuel cells reported recently.

2. Materials and Methods

2.1. Chemicals and reagents

Glucose oxidase (GOD), laccase and bovine serum albumin (BSA) were purchased from F. Hoffmann-La Roche, Ltd. GA, starch and glutaraldehyde were purchased from Sinopharm Chemical Reagent Co., Ltd. Starch solution was prepared by suspending suitable amount of starch powder into 0.1 M phosphate buffer under heat to boiling in microwave oven, which was cooled down at room temperature before use.

The specific enzymatic activity of GA is defined as the amount of glucose (in μmol) generated by 1 mg GA per minute in the excess of starch, while the specific enzymatic activity of GOD is defined as the amount of glucose (in μmol) consumed by 1 mg GOD per minute in the excess of glucose. Their activities were measured separately by spectrophotometry method, showing that 1 mg GA could generate 118 μmol glucose from starch per minute while 1 mg GOD could consume 297 μmol glucose per minute at the same condition.

2.2. Preparation of sequential biosensor

The sequential biosensor was fabricated on a glassy carbon electrode (GCE, diameter of 3 mm) which was polished to a mirror finish using 0.3 and 0.05 μm alumina slurry, followed by rinsing thoroughly with deionized water. After ultrasonic processing in anhydrous ethanol and ultrapure water, respectively, the electrode was rinsed with ultrapure water and dried at room temperature.

In preparation of sequential biosensor, 5 μL of multiwalled carbon nanotubes (MWNTs) suspension (2 mg MWNTs dispersed in 1 ml ultrapure water with ultrasonic processing) was dripped on the inverted GCE surface and dried in air. Next, different volumes of GOD solution (3000 U/ml) and GA solution (1200 U/ml), 2 μL of BSA (1% w/w) and 5 μL of glutaraldehyde (1% w/w) were mixed together on the inverted GCE to fabricate various modified electrodes and dried overnight at 4 °C in refrigerator. A glucose biosensor was also constructed with the similar method in which 6 μL of GOD solution (3000 U/ml), 2 μL of BSA and 5 μL of glutaraldehyde were applied.

2.3. Apparatus and electrochemical measurements

Electrochemical measurements were performed using a CHI660D potentiostat (CH Instruments, Chenhua, Shanghai, China). The electrochemical response was measured in a conventional three-electrode system using a chemically modified GCE as working electrode, a Pt wire auxiliary electrode and a saturated calomel electrode (SCE) as reference electrode. All potentials were reported in this context with respect to this reference. All measurements were performed at room temperature (~23 °C).

2.4. Preparation of biofuel

The one-compartment biofuel cell contained GA/GOD/MWNTs/GCE employed as the bioanode and the laccase/MWNTs/GCE as biocathode, which were assembled together in 5 ml of 0.5% (w/w) starch (pH 5.0) solution. In the biocathode fabrication, laccase was used as biocatalyst to catalyze oxygen reduction to water. To improve the bioelectrocatalysis efficiency of the laccase based biocathode towards O_2 reduction, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was used as a redox mediator.

3. Results and discussions

3.1. Construction of sequential biosensor

The sequential biosensor was constructed by co-immobilizing GA and GOD on the MWNTs-modified GCE by chemical crosslinking method, where glutaraldehyde and BSA was used as crosslinking and blocking agent, respectively (Fig. 1A). The thus-prepared bioelectrode is denoted as GA/GOD/MWNTs/GCE. Cyclic voltammograms (CVs) of different modified electrodes are shown in Fig. 1B. No redox peaks could be found for both GA/GCE and GA/GOD/GCE in the presence of starch (Fig. 1B, curves a, b). Only increased background current was observed at GA/MWNTs/GCE in the presence starch solution (Fig. 1B, curve c). A pair of well-defined redox peaks were clearly observed at GA/GOD/MWNTs/GCE in bare phosphate buffer (Fig. 1B, curve f), which meant the direct electron transfer between enzyme (GOD) and electrode was facilitated by MWNTs through the redox center FAD/$FADH_2$

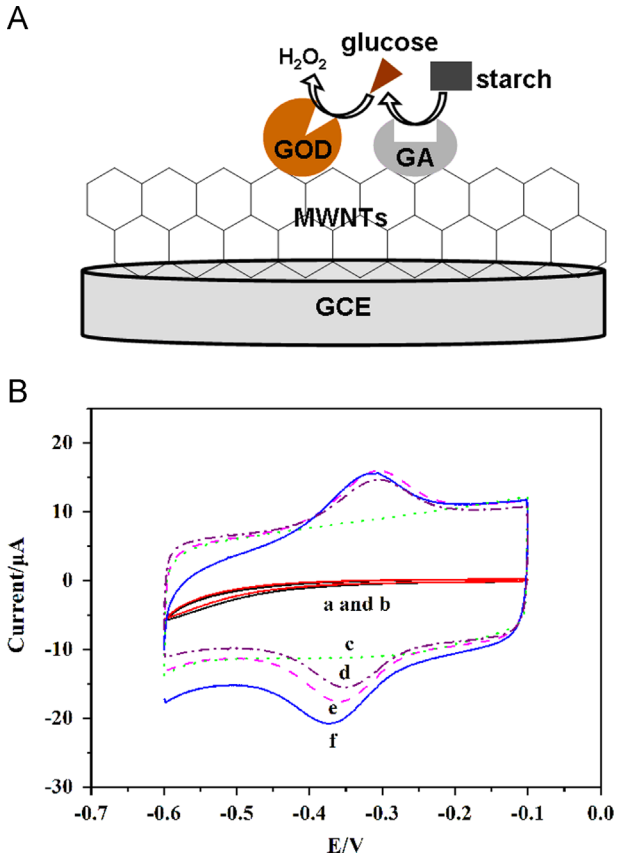

Fig. 1. (A) Schematic construction of sequential biosensor. (B) CVs of GA/GCE (a) and GA/GOD/GCE (b) in 0.05% (w/w) starch solution; CV of GA/MWNTs/GCE in the presence of 0.05% (w/w) starch solution (c); CVs of GA/GOD/MWNTs/GCE in the presence of 0.1% (w/w) glucose solution (d), in the presence of 0.05% (w/w) starch solution (e) and in bare 0.1 M phosphate buffer (pH 4.5) (f). Scan rate, 50 mV/s.

embedded in the GOD (Fig. 1A) (Artes et al., 2011; Li et al., 2013). Moreover, the cathodic peak current (i_{pc}) at −0.38 V decreased at the same GA/GOD/MWNTs/GCE in the presence of starch (Fig. 1B, curve e), suggesting that the GA catalyzed the hydrolysis of starch to glucose (reaction 1), the latter was further electrocatalytically oxidized by GOD immobilized on the electrode surface to gluconolactone in the presence of O_2, as schematically shown in Fig. 1A and reactions 2 and 3. Therefore, it is possible to detect starch using a sequential bioelectrode. On the other hand, GA/GOD/MWNTs/GCE exhibited the similar CVs in the presence of glucose or starch solution (Fig. 1B, curves d and e), which further confirmed the redox peaks were characteristic peaks of FAD/$FADH_2$. However, the peak potentials should be shifted a little with changing the substrate (starch or glucose) concentration (Fig. 1B, curves d and e). Additionally, the Δi_{pc} for GA/GOD/MWNTs/GCE in the presence of glucose had the similar trend as that value in the presence of starch, that is, for both glucose and starch at the same low concentration, both the Δi_{pc} values were roughly equal. Here the Δi_{pc} is defined as the difference of the cathodic peak current from CVs of the modified electrode in the presence of substrate and in the presence of bare buffer solution.

$$\text{Starch} \xrightarrow{GA} \text{Glucose} \quad (1)$$

$$\text{Glucose} + \text{GOx(FAD)} \rightarrow \text{Gluconolactone} + \text{GOx}(FADH_2) \quad (2)$$

$$\text{GOx}(FADH_2) + O_2 \rightarrow \text{GOx(FAD)} + H_2O_2 \quad (3)$$

3.2. Optimization of GA and GOD loading on response of the sequential enzyme electrode

The specific enzymatic activity of GA and GOD were measured to be 118 U/mg and 297 U/mg, respectively. In other words, the specific enzymatic activity ratio of GOD/GA was 2.5, suggesting that roughly, the catalytic rate of GOD was 1.5 times faster than that of GA in the beginning of reaction.

The loading of enzyme on electrode surface is a crucial parameter in the construction of enzyme biosensor, especially for sequential biosensor, of which the performance depends highly on the ratio of two enzymes (Zhou et al., 2001). For the convenient design of the sequential biosensor, the amounts of GA and GOD (in enzymatic activity unit) applied on the electrode surface should be optimized. In this study, the working electrode GA/GOD/MWNTs/GCE was prepared by loading both enzymes with different GA/GOD ratios, and CVs were measured in the presence of 0.5% (w/w) starch solution to investigate the change of the i_{pc} at about −0.38 V from the CVs. A series of modified electrodes were constructed with a constant loading of GOD (10 U) and varying GA loading ranging from 8.0 to 40 U. The Δi_{pc} increased with the GA amount in the cast film on the electrode ranging from 8 to 25 U (Supplementary material, Fig. S1A). Thereafter, the further increase in the GA loading induced the current decrease. So a GA (25 U) was loaded on the electrode for co-immobilization in the subsequent experiments. On the other hand, the GA/GOD/MWNTs/GCE based biosensor was fabricated with a constant 25 U of GA and varying GOD loading, and the responses of the prepared biosensors toward the electrocatalysis of 0.5% (w/w) starch solution were recorded separately. The dependence of Δi_{pc} value as a function of GOD loading is shown in Fig. S1B. Obviously, a maximal Δi_{pc} was achieved when 10 U of GOD was loaded. However, when the excess amount of two enzymes were loaded, the enzyme membrane became thick enough to block electron transfer between buffer solution and electrode surface (Li et al., 2013). Taken together, a loading of 25 U of GA and 10 U of GOD was applied for the preparation of the GA/GOD/MWNTs/GCE. Thus, the optimal GA/GOD loading ratio was 2.5, which is closely coincident with the specific enzymatic activity ratio of GA and GOD. Therefore, it is clear that the hydrolysis of starch by GA (reaction 1) was slower, which would become the rate-determining step in the sequential enzyme reaction, in agreement with the work reported by Vrbova's group (Vrbová et al., 1993).

3.3. Optimization of buffer pH on response of the sequential enzyme electrode

Considering that GA usually catalyzes starch in acidic solution, the pH-dependent enzymatic activity of GA was investigated within pH 3–7. The Δi_{pc} value increased sharply when buffer pH was changed from 3 to 4.5, thereafter Δi_{pc} decreased when the pH was higher than 5 (Supplementary material, Fig. S2). The largest Δi_{pc} value was achieved when the pH was 4.5, which was in accordance with previous report (Mishra and Debnath, 2002). Most enzyme-based biosensors were not stable in extreme pH condition (Vrbová et al., 1993; Torres et al., 2013), however, our bioelectrode could be stable in weak acidic buffer solution (pH 4.5) under continuous scans, suggesting the attractive feature of our sequential enzyme sensor.

3.4. Calibration curve of the sequential enzyme biosensor

The CVs of the GA/GOD/MWNTs/GCE in 0.1 M phosphate buffer (pH 4.5) containing different concentrations of starch were performed under the ambient-air condition (Supplementary material, Fig. S3). It should be mentioned here that our method to detect

starch is based on the measurement of O_2 consumption through the decrease of reduction peak. Obviously, from CVs, the Δi_{pc} at −0.38 V increased with the increasing concentration of starch (Supplementary material, Fig. S3). However, the changes in CVs are so miniscule at low starch concentration that might hardly serve for any differentiation. So amperometry was carried out in preparation of the calibration of the biosensor. The current–time curve was obtained with GA/GOD/MWNTs/GCE by using amperometry at an applied potential of −0.4 V (Fig. 2A). The current decreased after addition of starch solution and reached at 95% steady-state value within 10 s (Fig. 2A). The plot of the decreased current as a function of starch concentration is shown in Fig. 2B, from which the decreased current was linear with starch concentration within 0.005–0.7%, and thereafter, the current response was levelled off when the starch concentration was further added. So the linear range was 0.005–0.7%. The linear regression equation is $y=0.0008+1.015x$ with the coefficient $R=0.999$. The limit of detection (LOD) was estimated to be 0.003% starch ($S/N=3$). The linear range in our work was wider than those values obtained using trienzyme modified Clark-type oxygen sensors such as amyloglucosidase(AMG)/mutarotase(MUT)/GOD/catalase (CAT)-film/Clark-type oxygen sensor (0.01–0.4%) (Vrbová et al., 1993), AMG/MUT/GOD-film/Clark-type oxygen sensor (0.1–1%) (Watanabe et al., 1991) and AMG/MUT/GOD/Pd–Au/graphite (0.001–0.1%) (Abdul Hamid et al., 1990). The LOD in our case was little higher than 0.001% reported for the AMG/MUT/GOD/Pd–Au/graphite (Abdul Hamid et al., 1990), which was probably caused by the large current noise of amperometry during vigorous stirring.

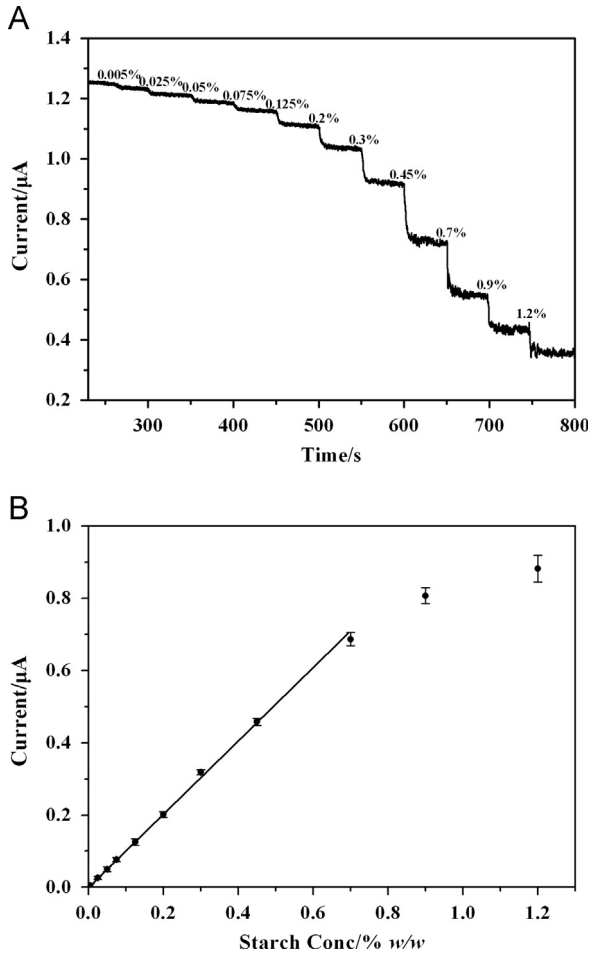

Fig. 2. (A) Current–time curve obtained at the GA/GOD/MWNTs/GCE on the successive addition of starch in 0.1 M phosphate (pH 4.5), on which the starch concentration denoted the starch concentration in the buffer. Applied potential, −0.4 V vs. SCE. (B) Typical calibration graph of the starch biosensor.

3.5. Selectivity of the sequential biosensor

The selectivity of the biosensor was investigated by comparing the current response of the bioelectrode on the successive addition of starch and various other substrates into the phosphate buffer when recording current–time curve at −0.4 V. As shown in Fig. 3, the successive addition of 0.1% starch resulted in obvious current decline (Fig. 3, arrows a, b and c). The presence of 0.04% D-glucose also exhibited current decrease (Fig. 3, arrow d), suggesting its good response to glucose. This is reasonable, because the sequential enzyme biosensor was fabricated with GA and GOD, where GOD is specific to glucose. The addition of other saccharides such as D-mannose, D-xylose, D-fructose, D-cellobiose and D-galactose as well as D-xylitol (each 0.2%) showed baseline, suggesting that the existence of these species did not affect the detection of starch. The addition of acetaminophen, ascorbic acid, and uric acid (each 10 mM) also showed no current change (Fig. 3, arrows k–m), suggesting that these common interfering species had no interference to the detection of starch. Therefore, this biosensor can be used to selectively detect starch without interference from common interfering species and other sugars except glucose. Actually, a GOD-modified electrode, which is constructed similarly to starch sequential biosensor, can be used to detect glucose before the measurement of starch using the sequential biosensor.

3.6. Operational stability of the sequential biosensor

Three different electrodes were prepared using the same procedure, and their CV responses were recorded in the same starch solution. A continuous measurement of CV was performed in a 0.1 M phosphate buffer containing 0.5% starch (pH 4.5). It was found that the peak currents for starch retained over 90% of the initial value after 200 continuous scans (Supplementary material, Fig. S4), which showed that the modified electrode had good operational stability (Chen et al., 2011).

3.7. Determination of starch in samples

The proposed biosensor was applied for real sample detection. Before measurement, suitable pretreatment of the samples should be performed. In a general procedure, an appropriate amount of

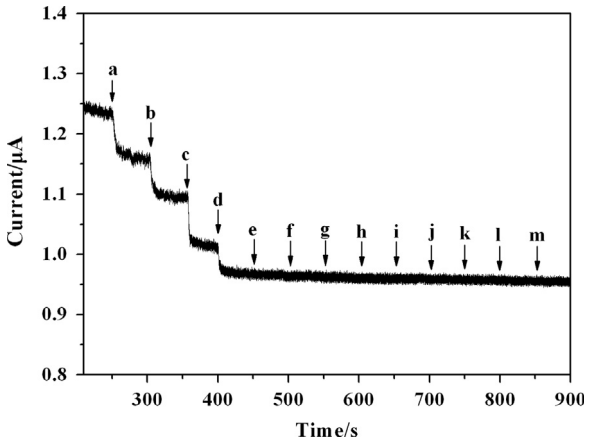

Fig. 3. Current–time curve obtained for the GA/GOD/MWNTs/GCE on the successive addition of 0.1% starch (a, b and c), 0.04% D-glucose (d), 0.5% D-mannose (e), 0.5% D-xylose (f), 0.5% D-xylitol (g), 0.5% D-fructose (h), 0.5% D-cellobiose (i), 0.5% D-galactose (j), 10 mM acetaminophen (k), 10 mM ascorbic acid (l), and 10 mM uric acid (m) in 0.1 M phosphate (pH 4.5).

Table 1
Determination of starch content in real samples.

Sample	Starch content (% w/w)	
	This work	Fehling's titration
Local snack	46.6 ± 0.4	50.0 ± 0.1
Kraft	37.5 ± 0.5	42.0 ± 0.1
Digestive pill	49.0 ± 0.3	51.0 ± 0.1
Local ham sausage	8.3 ± 0.1	9.0 ± 0.2
Banana	0.75 ± 0.07	0.80 ± 0.2

sample is freeze-dried and ground into tiny powder with mortar. Then the powder is washed repeatedly with anhydrous ethanol to remove any soluble saccharides, the precipitate is collected and dried in oven at 78 °C to remove the solvent ethanol. Subsequently, the dried precipitate is redissolved in 0.1 M phosphate buffer (pH 4.5) and filtered to remove any insoluble particles through a 0.22-μm membrane, and the filtrate is collected and aliquoted into 6 equal volumes. For 3 aliquots, the glucose+starch content in the sample solution is detected based on the established method. A GOD-modified electrode, which was constructed similarly to starch sequential biosensor, is used to determine the content of D-glucose in the other 3 aliquots. The contribution from the initial glucose contained in the samples before the process of GA hydrolysis is subtracted. The concentrations of the real samples were calculated based on the calibration graph multiplying the dilution ratios (Table 1). For comparison, Fehling's titration was carried out. Results obtained from sequential biosensor corresponded well to the results obtained by Fehling's titration (Table 1). Apparently, our method can be used to detect sample with lower starch content (such as banana), however, significant error was obtained with Fehling's titration. The starch contents obtained from our sequential sensor is systematically less than those values measured by Fehling's titration (Table 1). It is reasonable that Fehling's titration detected the total reduced saccharides in sample solution, whereas the sequential biosensor responded selectively to glucose. Further, Fehling's titration is usually involved in a lengthy hydrolysis process before titration, which is time-consuming. Taken together, our proposed method is advantageous over traditional Fehling's titration method.

3.8. Starch biofuel cell

As demonstrated above, sequential bioelectrocatalytic oxidation of starch (glucose) at GA/GOD/MWNTs/GCE makes it promising as a bioanode. Furthermore, direct bioelectrocatalytic oxidation of glucose at low potential starting at −0.4 V will be favorable for improving the open circuit voltage (OCV) of the BFC. In the current work, we developed starch/O_2 biofuel cell based on our recent work with modification (Xia et al., 2013).

The performances of the modified biocathode were examined in 0.1 M phosphate buffer (pH 5.0) containing 0.5% w/w starch. The OCV values varied between 0.45 V and 0.52 V depending on the amount of enzyme immobilized on the MWNTs/GCE. At the laccase/MWNT/GCE, an increased cathodic current appeared in the presence of O_2, while no cathodic catalytic current was observed under N_2 atomosphere (Fig. 4A). As seen from the polarization curves in Fig. 4A, the electrocatalytic reduction of O_2 started at about 0.52 V. To form a membrane-less starch/O_2 biofuel cell, this novel GA/GOD/MWNTs/GCE sequential enzyme bioanode was combined with the above described O_2 electroreducing cathode. The dependence of the power density on the operating voltage of the as-assembled biofuel cell in 0.5% (w/w) starch under O_2 is shown in Fig. 4B. The OCV of the as-assembled BFC was ca. 0.53 V and the maximum power density was 8.15 μW cm^{-2} at 0.31 V.

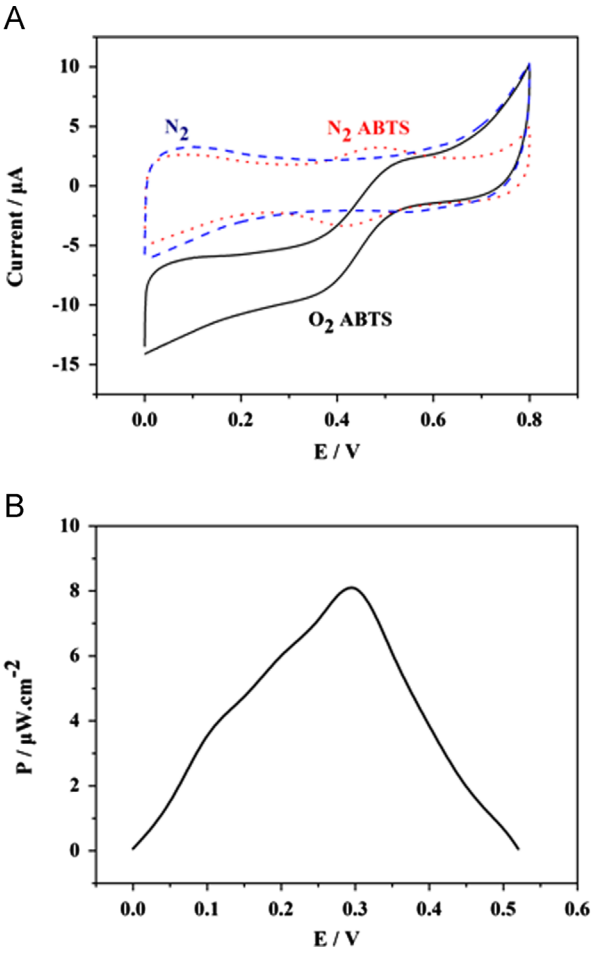

Fig. 4. (A), CVs of the laccase/MWNTs/GCE biocathode in 0.1 M phosphate buffer with 0.5% (w/w) starch (pH 5.0) under N_2-saturated atmosphere without ABTS (dashed line), and in presence of 0.5 mM ABTS under N_2-saturated (dotted line) and under oxygen-saturated atmosphere (solid line). (B) Dependence of the power density on the cell operating voltage of the starch/O_2 BFC.

Because the MWNT can facilitate the GOx catalysis (Li et al., 2013; Wang et al., 2013), the performance of this BFC was quite comparable to those of glucose/oxygen BFC reported recently (Li et al., 2009; Gao et al., 2011; Wen et al., 2011). To test the operational stability of the as-assembled BFC, the cell was operated continuously in a 0.5% w/w starch solution under ambient air. After 12 h operation, it retained 89% of its maximal power, suggesting a favorably stable power output process.

4. Conclusions

A novel electrochemical sequential biosensor GA/GOD/MWNTs/GCE was successfully constructed for the detection of starch. The proposed biosensor was based on the measurement of the decrease in the presence of starch, enabling to determine starch without the measurement of H_2O_2, thus simplified starch biosensor. The current linearly declined with the increasing concentration of starch ranging from 0.005% to 0.7% (w/w). The as-fabricated sequential biosensor can be applicable to the detection of the content of starch in snacks, pills and fruits, which were in good accordance with traditional Fehling's titration. Therefore, the proposed biosensor exhibited attractive features such as good operational stability, wide linear range and capable of determination of starch in real samples. Finally, the starch/O_2 biofuel cell was assembled using the GA/GOD/MWNTs/GCE electrode as bioanode

and laccase/MWNTs/GCE as biocathode, which exhibited open circuit voltage of ca. 0.53 V and the maximum power density of 8.15 $\mu W\ cm^{-2}$ at 0.31 V, comparable with the other glucose/O_2 based biofuel cells reported recently. This research provides a new paradigm for the investigation of other sequential enzyme systems.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (Nos. 91227116 and 21275152) and the Hundred-Talent-Project (No. KSCX2-YW-BR-7) and the Knowledge Innovation Project in Biotechnology (No. KSCX2-EW-J-10-6), Chinese Academy of Sciences. We are indebted to Prof. Dr. Gebo Pan in Suzhou Institute of Nan-Tech and Nano-Biomics, Chinese Academy of Sciences for gifting multi-walled carbon nanotubes.

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.2013.07.021.

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