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Biofuel cell and phenolic biosensor based on acid-resistant laccase–glutaraldehyde functionalized chitosan–multiwalled carbon nanotubes nanocomposite film

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ABSTRACT

To immobilize laccase (Lac) from *Trametes versicolor* that shows its maximum enzymatic activity in acidic aqueous solutions, the biopolymer chitosan (CS) was chemically modified with glutaraldehyde (GA) to form GA functionalized CS (GAfCS), which was then allowed to react with Lac to form a Lac–GAfCS composite that is robust in weakly acidic solutions (two-step protocol), as confirmed by quartz crystal microbalance and durability tests. The Lac–GAfCS–multiwalled carbon nanotubes (MWCNTs)/glassy carbon (GC) electrode exhibited good catalytic activity towards O_2 reduction in the presence of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS), and the pH-dependent enzymatic activity of the immobilized Lac towards O_2 reduction was examined. A glucose/air biofuel cell was fabricated, with the Lac–GAfCS–MWCNTs/GC electrode as the biocathode and a glucose oxidase (GOx)–GAfCS–MWCNTs/GC electrode as the bioanode in a Nafion membrane-separated acetate buffer solution (pH 5.0). The biofuel cell output a maximum power density of 9.6 μW/cm^2, an open-circuit cell voltage of 0.19 V, and a short-circuit current density of 114 μA/cm^2, respectively, as measured with an electrochemical noise (ECN) apparatus. Furthermore, the Lac–GAfCS–MWCNTs/GC electrode was applied to determine catechol in Britton–Robinson buffer solution (pH 3.0), with a linear range of 0.1–50 μM and a limit of detection of 20 nM. In comparison with the direct use of GA for one-pot Lac–GA-CS or Lac–GA crosslinking to immobilize Lac, the use of macromolecular GAfCS in the proposed two-step protocol was proven to be less harmful to the enzymatic activity and thus more suitable for immobilizing the enzyme to construct the biofuel cell and biosensor. This work may be helpful for exploiting the popular biocompatible CS as an acid-resistant film matrix for many other biotechnology applications, and the proposed two-step crosslinking protocol is recommended for high-activity immobilization of other biomolecules.

1. Introduction

Laccase (Lac) as a copper-containing oxidase is widely distributed in fungi (Luterek et al., 1997), higher plants (Gramss et al., 1999), and some bacteria (Alexandre and Zhulin, 2000), which can catalyze the oxidation of many phenolic and non-phenolic compounds accompanied by the reduction of oxygen. Lac has been applied to many industrial processes including decolourization of dyes (Abadulla et al., 2000), pulp delignification (Crestini and Argyropoulos, 1998), oxidation of organic pollutants in wastewater (Annibale et al., 1999; Lante et al., 2000), microbial transformation of natural products (Hosny and Rosazza, 2002), and the development of biosensors for determination of phenols and their derivatives in blood, wine, tea, fruit juice, and oil (Freire et al., 2002; Gomes et al., 2004; Jarosz-Wilkoazka et al., 2005; Torrecilla et al., 2007; Vianello et al., 2004, 2006; Zhang et al., 2007). Also, Lac can reduce oxygen directly to water in a four-electron transfer step without intermediate formation of hydrogen peroxide at the expense of oxidation of a variety of mediators, e.g., 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS). Thus, Lac has been widely used to catalyze the reduction of oxygen at the biocathode of some biofuel cells (BFC) (Chen et al., 2001; Brunel et al., 2007).

The effective immobilization of enzymes on the electrode is the key step to construct an enzyme electrode and a BFC. Chitosan (CS) is a popular biocompatible polysaccharide material that displays excellent film-forming ability, good adhesion, high mechanical strength, and susceptibility to chemical modifications due to the presence of reactive hydroxyl and amino functional groups (Huang et al., 2002; Zhang et al., 2004). It has been widely used as a support for immobilization of enzymes via electrodeposition (Luo et al., 2005; Zhou et al., 2007), crosslinking (Wei et al., 2002; Zhang et

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al., 2004), and dip-coating (Liu et al., 2006). However, the protonation/deprotonation of CS ($pK_a \approx 6.1$–7.0) make CS soluble/insoluble below/above ca. pH 6 (Zhou et al., 2007), thus the biosensor with a CS film as the enzyme-immobilization matrix should be employed at solution pH sufficiently above its pK_a (Liu et al., 2006; Zhou et al., 2007), and the applicability of CS material for biosensing in acidic solutions is still a challenge to date. Carbon nanotubes (CNTs) are a kind of relatively new nanomaterials that displays attractive structural, mechanical, and electronic properties, including improved electrochemical activity (Tasis et al., 2006). The CNTs have been widely employed as nanowires to facilitate the electron transfer of a variety of enzymes with the electrode in the emerging nanobiosensing field (Luong et al., 2004; Zhao et al., 2004). The CS–CNTs nanocomposite has also been used as an excellent enzyme-immobilization matrix for biosensing in neutral aqueous solution (Zhou et al., 2007; Zhang et al., 2004; Liu et al., 2006).

Lac has been immobilized on CNTs (Karnicka et al., 2008; Liu and Dong, 2008) and CS microspheres (Fernandesa et al., 2008) for phenolic biosensing and bioelectrocatalytic reduction of oxygen. The CS–CNTs nanocomposite has also been utilized to immobilize Lac from *coriolos versicolor* in the absence of a crosslinking reagent, and the Lac incorporated into the composite film remained better catalytically active than that dissolved in solution at pH 6 (Liu et al., 2006). The different catalytic activity between free and immobilized Lac has been ascribed to the better microenvironment of the CS-immobilized enzyme (Yang et al., 2006). However, a lower pH is still helpful for most Lac from fungi to achieve the largest catalytic activity (Mano et al., 2002; Liu et al., 2006; Brunel et al., 2007), and it is thus of obvious interest and significance to develop a Lac-CS–CNTs nanocomposite film to stably work in acidic solutions via suitable chemical modifications.

Herein, CS is chemically modified with glutaraldehyde (GA) to develop GA functionalized CS (GAfCS), which is then allowed to react with Lac, and the electrode-supported Lac–GAfCS film so prepared becomes sufficiently stable in a weakly acidic solution, as confirmed by quartz crystal microbalance (QCM) and durability tests. The Lac–GAfCS–multiwalled CNTs (MWCNTs)/glassy carbon (GC) electrode is developed and used in a glucose/air BFC and in catechol biosensing with good performance.

2. Experimental

2.1. Chemicals

Lac from *Trametes versicolor* (20 U/mg), glucose oxidase (GOx) (~150 U/mg), Nafion 117, and ABTS were purchased from Sigma–Aldrich. CS from crab shells (90% deacetylated) was purchased from Sinopharm Chemicals Co., Ltd. (Shanghai, China). MWCNTs (20–40 nm diameter) were purchased from Shenzhen Nanotech Port Co. The MWCNTs were purified and acidified prior to use by stirring them in 2 M aqueous nitric acid for 20 h. Ferrocene monocarboxylic acid (FCMA), catechol, GA, and other chemicals were of analytical grade. A Nafion 117 proton-exchange membrane (ca. 150 μm thickness) was prepared by dropping 0.5 mL of 5 wt% Nafion (in a mixture of lower aliphatic alcohols and water) on a clean and flat glass, and drying in air. Acetate buffer solution (pH 5.0) was 0.10 M HAc–NaAc aqueous solution. Britton–Robinson (B–R) buffer solution (pH 3.0) was prepared with equimolar amounts (0.040 M) of acetic, phosphoric, and boric acids, adjusted to the desired pH with NaOH. Milli-Q ultrapure water (Millipore, ≥18 MΩ cm) was used throughout.

2.2. Apparatus

All electrochemical experiments were performed on an Autolab PGSTA 30 electrochemical workstation (Eco Chemie BV, The Netherlands) with the GPES 4.9 software. QCM studies were carried out with a computer-interfaced HP4395A impedance analyzer. AT-cut 9-MHz gold-coated piezoelectric quartz crystals (PQCs, 12.5 mm diameter, Model JA5, Beijing Chenjing Electronics Co., Ltd., China) were used. The gold electrode of 6.0 mm diameter on one side of the PQC was exposed to the solution, while the other side of the PQC faced air. A GC disk electrode of 3.0 mm diameter, a KCl-saturated calomel electrode (SCE), and a platinum plate served as the working, reference, and counter electrodes, respectively, in the electrochemical experiments. All potentials herein are referenced to SCE. The cell voltage (V_{cell}) and the cell current (I_{cell}) of the glucose/air BFC at varying external loads (R_e) were dynamically monitored with the electrochemical noise (ECN) module of the Autolab PGSTA 30 electrochemical workstation, the details of which were recently reported (Tan et al., 2008). All experiments were done at room temperature (25 ± 2 °C) under air atmosphere.

2.3. Preparation of the GAfCS

A stock solution of 0.50 wt% CS was prepared by dissolving accurately weighed solid CS in HAc and then buffered to pH 5.0 with NaOH. GAfCS was prepared by drop-by-drop addition of 10 mL 0.50 wt% CS solution into 10 mL 50% GA under magnetic stirring and then allowed to react for 48 h at room temperature. To confirm only one aldehyde group of GA reacting with amino groups of CS, a high molar ratio of GA to CS glucosamine units was used here (Wei et al., 2002). The reaction involved formation of Schiff base structures (Roberts and Taylor, 1989; Wei et al., 2002),

$$\mathrm{CS{-}(NH_2)_{\mathit{n}} + \mathit{n}O{=}CH{-}(CH_2)_3{-}CH{=}O \rightleftharpoons CS{-}(N{=}C{-}CH_2{-}CH_2{-}CH_2{-}CH{=}O)_{\mathit{n}} + \mathit{n}H_2O} \quad (1)$$

In comparison with pure GA and CS, the GAfCS displayed FT-IR peaks at 1634 and 1718 cm^{-1} (Fig. 1S in the Supplementary Data), which can be assigned to the stretching vibrations of the C=N groups and the tethered aldehyde groups, respectively. Also, the mixed solution after the GAfCS reaction for 48 h became yellow, implying the incorporation of chromophore in the GAfCS. The unreacted GA was removed from the solution by dialyzing it for 36 h against pH 5.0 acetate buffer. The prepared solution of 0.20 wt% GAfCS was stored in a refrigerator (4 °C).

2.4. Preparation of enzyme electrodes

The GC electrode was polished with 1.0 and 0.05 μm alumina slurry sequentially and then washed ultrasonically in water and ethanol for 15 min, respectively. Then, the GC electrode was subject to potential cycling (−0.2 to 1.0 V, 10 mV s^{-1}) in 0.20 mol L^{-1} aqueous $HClO_4$ until reproducible cyclic voltammogram was obtained.

The Lac–GAfCS/GC electrode was fabricated as follows. 10 μL of 0.20 wt% GAfCS solution was well mixed with 10 μL of 2.0 mg/mL aqueous Lac for 60 s, then 5 μL mixture was cast at the GC electrode and air-dried at 4 °C for 6 h. Also, 10 μL of this liquid mixture was cast on the QCM Au electrode and air-dried for QCM examination into the film stability, which resulted in a dry frequency decrease by ca. −15 kHz. The Lac–GAfCS film exhibited a much stronger FT-IR peak at 1634 cm^{-1} but a much weaker peak at 1718 cm^{-1} than GAfCS, resulting from the conversion of C=O to C=N groups after the Lac–GAfCS reaction (Fig. 1S in the Supplementary Data). It should be noted that the mixed solution gelled after ca. 2 min due to the Lac–GAfCS reaction.

The Lac–GAfCS–MWCNTs/GC electrode was fabricated as follows. 1.0 mg MWCNTs was well dispersed in 1 mL of 0.20 wt% GAfCS via sonication for 1 h. 1.0 mg MWCNTs was chosen here, because a higher content of MWCNTs decreased the film adhesion

to the electrode surface, and a lower content of MWCNTs decreased the biocatalytic current response. 10 μL GAfCS–MWCNTs was then mixed with 10 μL of 2.0 mg/mL Lac. 5 μL mixture was cast on the GC electrode and air-dried at 4 °C for 6 h. To construct the bioanode for the glucose/air BFC, a GOx–GAfCS–MWCNTs/GC electrode was similarly fabricated, except for the replacement of 2.0 mg/mL Lac with 2.0 mg/mL GOx.

The Lac–GA-CS–MWCNTs/GC electrode was prepared for comparison. 10 μL of 5% GA was well mixed with 10 μL solutions of 2.0 mg/mL Lac, 0.20 wt% CS and 1.0 mg/mL MWCNTs. 5 μL mixture was cast at a GC electrode and air-dried at 4 °C for 6 h. Also, the Lac–GA-CS/GC electrode was similarly fabricated, except for the absence of MWCNTs.

The Lac–GA–MWCNTs/GC electrode was also prepared for comparison. 10 μL solution of 5% GA was well mixed with 10 μL solutions of 1.0 mg/mL MWCNTs and 2.0 mg/mL Lac. Then, 5 μL mixture was cast at a GC electrode and air-dried at 4 °C for 6 h. Also, the Lac–GA/GC electrode was similarly fabricated, except for the absence of MWCNTs.

All modified film electrodes were stored in pH 5.0 acetate buffer at 4 °C when not in use.

2.5. QCM and electrochemical measurements

The resonant frequency (f_0) and the motional resistance (R_1) of the Lac–GAfCS modified QCM Au electrode were monitored in pure water, pH 5.0 and 4.0 acetate buffers, and pH 3.0 B–R buffer for film-stability tests.

The setup of the fabricated glucose/air BFC is schematically shown in Fig. 2S in the Supplementary Data. The anode and cathode compartments (20 mL capacity for each) were separated by the prepared Nafion 117 ion-exchange membrane, and the distance between the anode (GOx–GAfCS–MWCNTs/GC) and the cathode (Lac–GAfCS–MWCNTs/GC) was fixed at 13 cm for convenience. The anolyte was 20 mL stirred acetate buffer (pH 5.0) containing 10 mM glucose and 0.50 mM FCMA, and the catholyte was 20 mL stirred acetate buffer (pH 5.0) containing 1.0 mM ABTS.

Typical steady-state current responses of the enzyme electrodes were obtained at 0.05 V by successive injections of catechol into 10 mL B–R buffer solution (pH 3.0) under magnetic stirring.

3. Results and discussion

3.1. Characterization of Lac–GAfCS–MWCNTs/GC

First of all, we examined the stability of the Lac–GAfCS film in acidic solutions via the QCM technique, which can detect minute changes in mass loading and viscoelasticity for a foreign film on the electrode surface (Martin et al., 1991; Xie et al., 1999). As shown in Fig. 1, the f_0 and R_1 responses were very stable in water. The additions of acetate buffers (pH 4.0 and 5.0) suddenly decreased f_0 and simultaneously increased R_1, then they became very stable, and the values of $-\Delta f_0/\Delta R_1$ of ca. 10 Hz/Ω should indicate viscous effect-induced responses of f_0 and R_1 here (Xie et al., 1999). The larger transient responses of f_0 and R_1 to pH 4.0 than those to pH 5.0 may result from the more viscous HAc molecules at pH 4.0. Also, both f_0 and R_1 kept very stable for a long time after the addition of the pH 3.0 B–R buffer, indicating that the Lac–GAfCS film is highly stable in the weakly acidic media, and the GA coupling to form mutilayered network film structure can enhance the mechanical stability of enzyme films (Freire et al., 2001; Rogalski et al., 1999; Kharitonov et al., 2000).

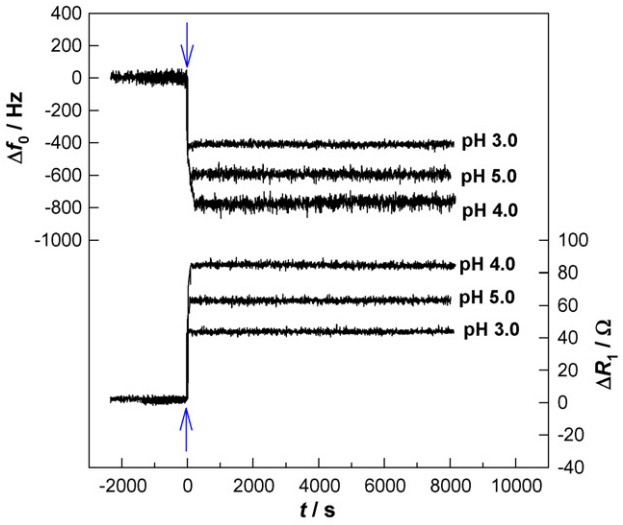
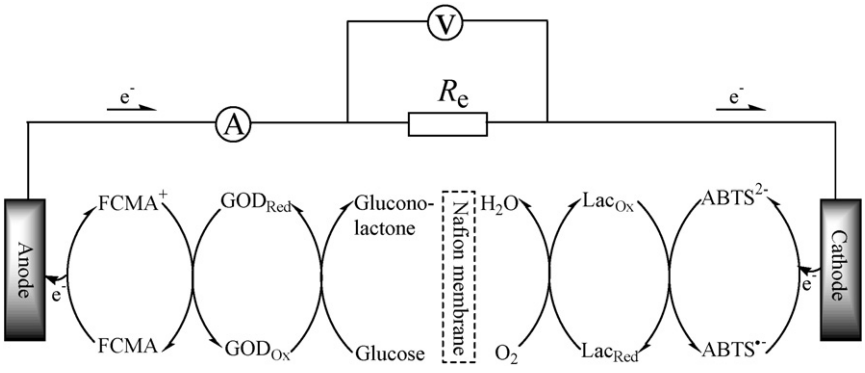


Fig. 1. QCM responses at a Lac–GAfCS/Au electrode to the addition of 10 mL pH 5.0 acetate buffer, 10 mL pH 4.0 acetate buffer (each finally to 0.20 mol/L HAc–NaAc), or 10 mL pH 3.0 B–R buffer into stirred 10 mL water. The added B–R buffer contained 0.040 M acetic, phosphoric, and boric acids, adjusted to pH 3.0 with NaOH. Arrows indicate the addition moments.

The electroactive ABTS has been used as a solution mediator for some enzymes (Tayhas et al., 1999; Tsujimura et al., 2001). Cyclic voltammetry was conducted here to examine the electrochemistry of ABTS at a bare GC electrode in nitrogen saturated B–R buffer solution (pH 3.0) containing 1.0 mM ABTS (Fig. 3S in the Supplementary Data). A pair of well-defined redox peaks of ABTS centering at 0.46 V was observed. Both anodic and cathodic peak currents were proportional to the square root of scan rate between 10 and 500 mV/s. The peak-to-peak separation ($\Delta E_p = E_{pa} - E_{pc}$) was 0.11 V at each scan rate, and E_{pa} and E_{pc} also changed negligibly at these scan rates, as reported by Tayhas et al. (1999). As shown in Fig. 4S in the Supplementary Data, the peak-to-



Scheme 1. Electron transfer steps at the cathode and the anode of the glucose/air BFC.

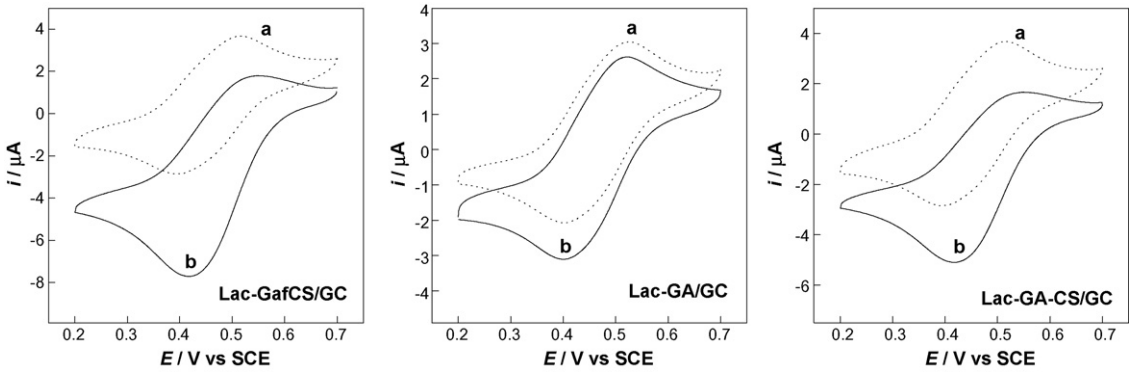


Fig. 2. Cyclic voltammograms (second cycle) of several enzyme electrodes in nitrogen (a) or air (b) saturated B–R buffer (pH 3.0) containing 1.0 mM ABTS. Scan rate: 10 mV/s.

peak separation changed little but the peak currents increased at Lac–GAfCS/GC, due probably to the enrichment of anionic $ABTS^{2-}$ in the film (curve b). However, the peak currents increased largely at Lac–GAfCS–MWCNTs/GC (curve c), resulting from the increased electroactive area after the incorporation of conducting MWCNTs.

An important property of Lac is to catalyze oxidation of substrates coupled with the electrocatalytic reduction of oxygen to water in a four-electron process without producing hydrogen peroxide. In the catalysis cycle of Lac in an oxygen-containing solution (Scheme 1), the Lac is oxidized by oxygen and can be turned over by the mediator ABTS with production of $ABTS^{\bullet+}$, and the ABTS can be regenerated at the cathode, thus the whole catalysis cycle solely consumes oxygen. Fig. 2 shows the cyclic voltammograms of several enzyme electrodes in air or nitrogen saturated B–R buffer solution (pH 3.0) containing 1.0 mM ABTS. The increase in the reduction peak current (oxygen vs. nitrogen saturation, defined as the catalytic current here) resulted from the electroreduction of the enzymatically generated $ABTS^{\bullet+}$ as the oxidized state of ABTS. The catalytic current of 4.9 μA at Lac–GAfCS/GC was larger than that at GA–Lac/GC (1.1 μA) or Lac–GA-CS/GC (2.2 μA). This finding demonstrates that the proposed two-step crosslinking protocol is more favorable for the preservation of enzyme activity, since the GAfCS with only one-end active aldehyde groups pending can less influence the enzyme configuration during enzyme immobilization, but the intramolecular enzyme crosslinking in the GA–Lac and Lac–GA-CS cases may take place to some degree to decrease the enzyme activity. As expected, the incorporation of conducting MWCNTs (Lac–GAfCS–MWCNTS/GC) maximized the catalytic current (7.0 μA at pH 3.0, upper right panel of Fig. 3).

The pH effect on the amperometric response at Lac–GAfCS–MWCNTs/GC is shown in Fig. 3. The catalytic current at pH 3.0 (7.0 μA) was larger than that at pH 2.0 (5.7 μA) or pH 5.0 (3.8 μA), and little catalytic response was observed at pH 7.0, indicating that the Lac here shows its largest catalytic activity at pH 3.0, as reported previously (Tayhas et al., 1999).

3.2. The glucose/air BFC with the Lac–GAfCS–MWCNTs/GC biocathode

Electron transfer steps in the glucose/air BFC with the bioanode mediator of FCMA and the biocathode mediator of ABTS are shown in Scheme 1. A pair of redox peaks centering at 0.28 V was observed on the GOx–GAfCS–MWCNTs/GC bioanode in acetate buffer (pH 5.0) containing 0.50 mM FCMA (Fig. 5S in the Supplementary Data), and an obviously larger anodic catalytic current was observed after adding 10 mM glucose. Also, the anodic catalytic current, which is virtually characteristic of the catalytic activity of GOx, became maximum at pH 7.0 (Insert of Fig. 5S). The catalytic activity of Lac from *T. versicolor* towards O_2 reduction increased but that of GOx towards glucose oxidation decreased with pH decrease from 7.0 to 3.0 (Brunel et al., 2007; Tan et al., 2008; Tayhas et al., 1999; Liu et al., 2005), so most of the glucose/O_2 BFCs were performed at pH 5.0 for convenience. Obviously, the anodic catalytic current at GOx–GAfCS–MWCNTs/GC was much larger than the cathodic catalytic current at Lac–GAfCS–MWCNTs/GC (Fig. 2D), since the latter was limited by the concentration of air-saturated soluble oxygen. Thus, I_{cell} of the glucose/air BFC should be mainly dependent on the biocathode rather than the bioanode.

The formal potentials of FCMA and ABTS are 0.28 and 0.46 V, respectively, so the open-circuit cell voltage should be ca. 0.18 V. Fig. 4 shows V_{cell} and I_{cell} of the BFC operating at varying R_e. As R_e increased, V_{cell} increased and reached the largest value of 0.19 V at R_e = 200 kΩ, while I_{cell} decreased and reached almost zero also at R_e = 200 kΩ, with a short-circuit current density (j_{cell}) of 114 μA/cm^2 (electrode geometric area = 0.07 cm^2). Fig. 4 also shows j_{cell} and the power density (P) of the BFC as functions of V_{cell} at these R_e. The maximum P was 9.6 μW/cm^2, being larger than those of the glucose/air BFCs reported by Katz et al. (1999) (5.1 μW/cm^2) and Tan et al. (2008) (8.0 μW/cm^2), but lower than that of the glucose/air BFC based on a diaphorase/glucose dehydrogenase double layer-coated anode and a polydimethylsiloxane-coated Pt cathode (14.5 μW/cm^2) (Sato et al., 2005). Virtually, replacing the planar electrode with the microfiber electrode to increase the active surface area (Chen et al., 2001; Mano et al., 2002), and replacing air-saturated solution with oxygen-saturated solution to increase the oxygen concentration (Brunel et al., 2007; Liu et al., 2005), were reported to be efficient in enhancing the maximum P, and these protocols might be adopted after appropriate modifications to improve our BFC in the future. The internal resistance of the present BFC is calculated to be ca. 14 kΩ from the slope of $V_{cell} - j_{cell}$ curve at small j_{cell}, resulting mainly from the long anode-to-cathode distance and the isolation of a Nafion membrane. The long-term discharging of the BFC was dynamically monitored for 15,000 s with the ECN device (Fig. 6S in the Supplementary Data). After the continuous operation for 15,000 s at 20 kΩ R_e, the V_{cell} and I_{cell} decreased to 0.108 V and 5.4 μA from their initial values of 0.116 V and 5.8 μA, respectively, both being 93% of their initial values, indicating that the present glucose/air BFC worked highly stably, and the GAfCS–MWCNTs composite is an appropriate enzyme-immobilization matrix for the development of BFC.

3.3. Phenolic biosensing

Phenols and their derivatives are widely distributed in natural products, but some toxic phenols and their derivatives from industry are recently attracting serious environmental concern. The detection of phenolic compounds is another important applica-

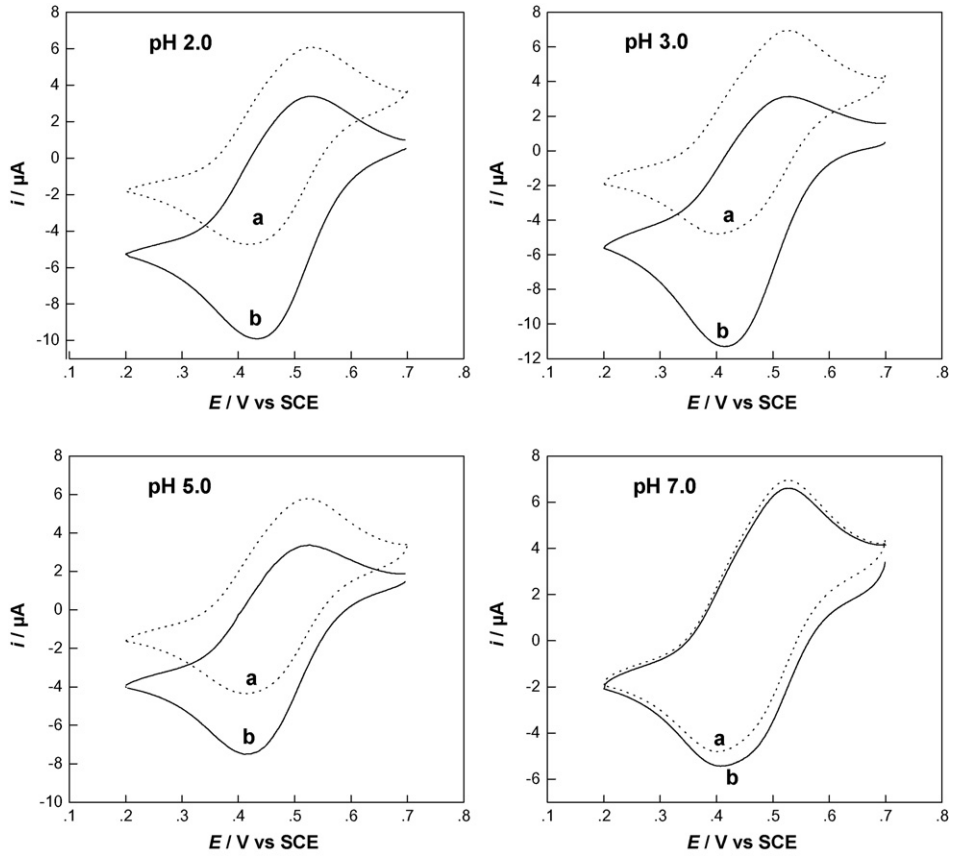


Fig. 3. Cyclic voltammograms (second cycle) at Lac–GAfCS–MWCNTs/GC in nitrogen (a) or air (b) saturated B–R buffer containing 1.0 mM ABTS at different pH. Scan rate: 10 mV/s.

tion fields for the Lac modified electrodes. Catechol is used herein as the model phenolic substrate to be detected. To achieve the largest enzymatic activity of Lac, the solution pH of 3.0 and the applied potential of 0.05 V (optimized) were selected to achieve the maximum biosensing sensitivity for catechol determination in subsequent experiments.

Fig. 5 shows the steady-state current responses at Lac–GAfCS–MWCNTs/GC, Lac–GA-CS–MWCNTs/GC, or Lac–GA–MWCNTs/GC at 0.05 V to the successive additions of catechol into pH 3.0 B–R buffer. The biosensing performance is given as follows, linear response range (LRR) of 0.1–50 μM with linearity regression equation (LRE) of ΔI (μA) = −0.040 −101.9c (mM) (r^2 = 0.9996), and a limit of detection (LOD) of 20 nM (S/N = 3) at Lac–GAfCS–MWCNTs/GC; LRR of 0.3–100 μM with LRE of ΔI (μA) = −0.0428 −49.7c (mM) (r^2 = 0.9985), and an LOD of 100 nM at Lac–GA-CS–MWCNTs/GC; LRR of 1–190 μM with LRE of ΔI (μA) = −0.228 −24.0c (mM) (r^2 = 0.9985), and an LOD of 800 nM at Lac–GA–MWCNTs/GC. Obviously, the sensitivity at Lac–GAfCS–MWCNTs/GC is higher than those at Lac–GA-CS–MWCNTs and Lac–GA–MWCNTs/GC, being probably due to the better maintenance of the enzymatic activity of the immobilized Lac in the former case. The 20 nM LOD at Lac–GAfCS–MWCNTs/GC is much lower than the reported values of 2 μM (Yaropolov et al., 1995) and 0.66 μM (Liu et al., 2006).

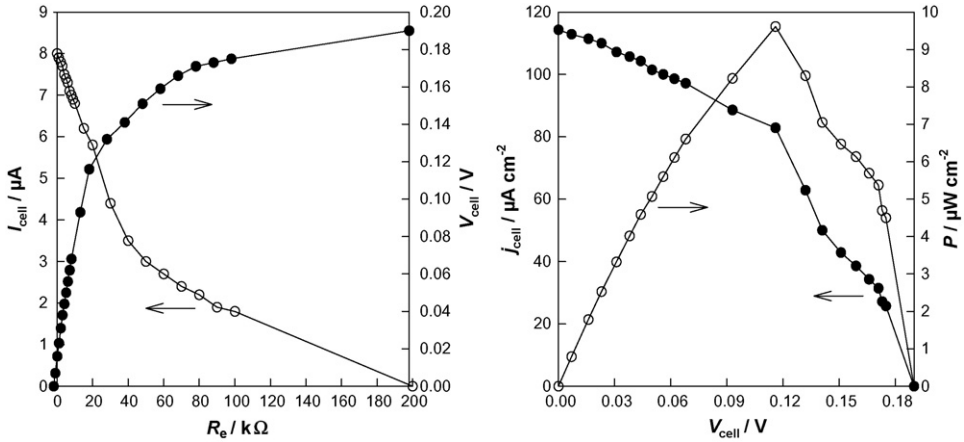


Fig. 4. V_{cell} and I_{cell} as functions of external load (R_e = 0–200 kΩ) for the glucose/air BFC as well as j_{cell} and P as functions of V_{cell}.

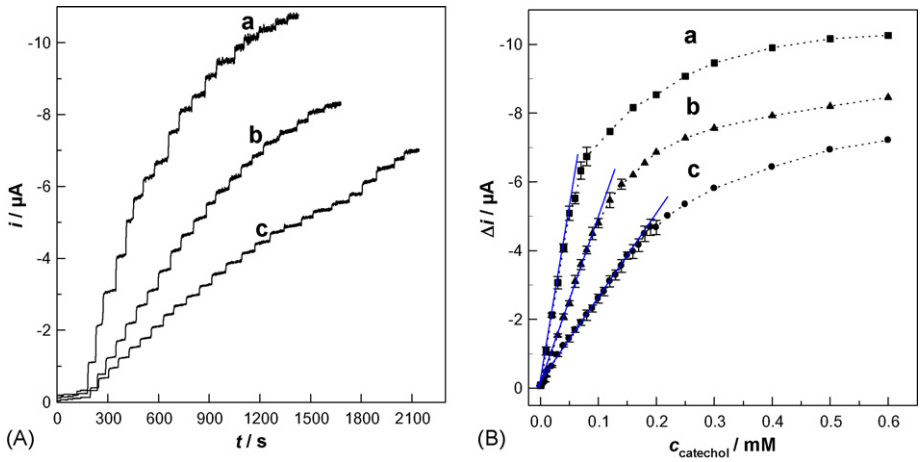


Fig. 5. (A) Time-dependent current responses to successive injections of catechol into stirred B–R buffer (pH 3.0) and (B) the calibration curve at Lac–GAfCS–MWCNTs/GC (a), Lac–GA-CS–MWCNTs/GC (b), or Lac–GA–MWCNTs/GC (c). Applied potential: 0.05 V vs. SCE.

Michaelis–Menten kinetic analysis is carried out using the Lineweaver Burk plot, which states that:

$$\frac{1}{\Delta i_{ss}} = \frac{1}{\Delta i_{max}} + \frac{K_m^{app}}{\Delta i_{max}} \frac{1}{c} \quad (2)$$

where Δi_{ss} is the steady-state current response after catechol addition, Δi_{max} is the maximum current response at saturated substrate concentration, c is the concentration of catechol, and K_m^{app} is the apparent Michaelis–Menten constant.

The K_m^{app} values were obtained to be 60 μM at Lac–GAfCS–MWCNTs/GC, 95 μM at Lac–GA-CS–MWCNTs/GC, and 170 μM at Lac–GA–MWCNTs/GC, respectively. Obviously, the smaller K_m^{app} at Lac–GAfCS–MWCNTs/GC indicates the greater bioaffinity of the immobilized Lac to catechol than those in the other two cases. The K_m^{app} values for Lac from *T. versicolor* were reported to be 70 μM (carbodiimide and glutaraldehyde co-crosslinking) (Portaccio et al., 2006) and 610 μM (glutaraldehyde crosslinking) (Freire et al., 2001), also validating the Lac–GAfCS–MWCNTs nanocomposite as a better matrix for Lac immobilization.

The operational stability at Lac–GAfCS–MWCNTs/GC was conducted at 10 μM catechol level, and continuous 20 determinations gave current responses of 1.05 μA with 3.03% RSD, as implied in Fig. 5. The storage stability of this enzyme electrode was studied by regular catechol detection experiments in a month, as shown in Fig. 7S in the Supplementary Data. After a week the biosensor retained 92% of its initial response. With the experiment prolonged, the further decrease of the response current was observed, and it remained about 85% of its initial response after a month.

4. Conclusions

In summary, a glucose/air biofuel cell and a phenolic biosensor with good performance have been constructed based on the synthesized acid-resistant Lac–GAfCS–MWCNTs nanocomposite film. The use of macromolecular GAfCS to immobilize enzyme has been proven to be less harmful to enzymatic activity and thus more suitable for immobilizing Lac to construct the biofuel cell and the biosensor, as compared with the direct GA crosslinking immobilization of Lac. The proposed two-step crosslinking protocol capable of retaining high activity of the immobilized biomolecules and operation in acidic media is expected to find wide biosensing applications.

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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.2008.11.026.

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