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Immobilization of Laccase into Poly(3,4-Ethylenedioxythiophene) Assisted Biocathode for Biofuel Cell Applications

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Reduction of dioxygen catalyzed by laccase was studied at poly(3,4-ethylenedioxythiophene) (PEDO) modified electrodes. PEDOT with laccase are directly fabricated on pt electrode by simple process. The cyclic voltammetry (CVs) has been used for the measurement of electroanalytical properties of analytes by means of modified electrodes. The GOx/MWCNTs modified electrode exhibited good catalytic activity towards glucose oxidation and the PEDOT-laccase/pt modified electrode exhibited good catalytic activity towards O_2. The maximum power density was 102 μWcm^{-2}. The stabilities of laccase, the reduction potentials and ratios of catalytic to background currents were compared. Efforts are underway to improve the interface transfer to achieve higher potential and current output.

Keywords: Laccase, PEDOT, Biofuel Cell, Bionanotechnology, Modified electrodes, Bioelectrocatalysis, Bioelectrochemistry, electrochemical.

1. INTRODUCTION

Biological fuel cells (BFCs) used to transform the chemical energy into electrical energy employ enzymes as catalysts and natural compounds e.g. glucose or ethanol, as the fuels [1-8]. Enzymatic biofuel cells employ enzymes to catalyze chemical reactions, thereby replacing traditional electrocatalysts present in conventional fuel cells. These systems generate electricity under mild conditions through the oxidation of renewable energy sources without greenhouse gas emissions or

environmental pollution. Enzymatic biofuel cells have attracted attention as alternative, cheap and environmentally friendly power sources that also could be implanted into living body [9]. BFCs can utilize glucose and dioxygen dissolved in the body fluids as fuel and oxidant, respectively, and could serve as power source for implanted devices such as: microvalves, drug dispensers, pacemakers, sensors, etc. [10]. The advantage of biofuel cells lies in the aspects of enzymatic catalysis namely, activity at near-room temperature, neutral pH and selectivity. However, enzymatic biocatalytic assemblies on electrode surfaces usually do not achieve significant electron transfer between the enzyme and the conductive support, mostly because of the electrical insulation of the biocatalytic site by the surrounding protein shells [11]. In general, redox mediators are introduced to shuttle electrons between the enzyme active site and the electrode surface [12]. To facilitate electron transfer between enzyme and electrode various mediators are usually employed [13-15]. Methods based on direct electron transfer would, however, be cheaper and much more convenient in practical applications.

Laccase (EC 1.10.3.2) has been extensively used as cathodic catalyst in enzymatic fuel cells due to its high redox potential (400 mV to 800 mV vs. NHE) [16-18]. It belongs to the multicopper oxidase (MCO) family which utilize a minimum of four copper ions to catalyze the four-electron reduction of O_2 to water, without release of H_2O_2. The copper ions of these enzymes are classified into three types depending on their optical and magnetic properties [19]. The first type (T1), so called blue copper, which is a primary acceptor of electrons from a substrate, and the second type (T2) and third type (T3) being a binuclear copper site) [20]. Type 1 (T1) copper has an intense Cys to Cu(II) charge transfer absorption band around 600 nm. This copper center accepts electrons from the electron-donating substrate, e.g. phenols [21-22] or metal ions, and relays these to the O_2 reduction site. From T1 electrons are transferred to the T2–T3 copper cluster, where transformation of O_2 to water occurs. For the dioxygen bioelectrocatalytic reduction catalyzed by laccase, mediators are typically used to facilitate transfer of electrons between the electrode and the active site of the redox center, T1, hidden inside the protein in the hydrophobic pocket arranged by amino-acid residues. 2,20-Azino- bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) has been widely recognized as an effective mediator to transfer electron from electrode to laccase [23].

Recent efforts have focused on increasing the stability of the biocatalytic films through coupling to surface modified electrodes. For example, diazonium salt chemistry can introduce functional groups to electrode surfaces for covalently anchoring of enzymes [24], mediator [25-26] or co-immobilized redox polymer and enzyme [27-29] or DNA [30]. The most common ones aim to stabilize the enzyme itself by immobilization. To increase the lifetime of enzymatically catalyzed biofuel cell or biosensor electrodes, several strategies and ideas have been published [31]. Immobilization of laccase on electrodes has allowed measuring high current densities of O_2 reduction at low overpotentials under optimum conditions of its enzymatic activity, overcoming the classic chloride inhibition limitation [32-33]. Several novel immobilization methods have been applied, such as entrapment of the enzyme in conducting ink, polymer–carbon nanotube mixture [34-39] or redox hydrogels [40-41], chemical bonding [42-43] and crosslinking [44-46]. Conducting polymers (CP), such as polyaniline, polypyrrole and poly(3,4-ethylenedioxythiophene) (PEDOT), have also widely been used as immobilization matrix for enzymes or mediator compounds [47-51]. Enzymes are usually

immobilized in conducting polymers by physical entrapment but sometimes covalent attachment is also used [52-55].

Conducting polymers have emerged as matrix material for model membranes or film [56], adding their potential to the research of biological cell membranes alongside and even in combination with the more traditional approach using artificial phospholipid bilayers [57-58]. Both types of these model membranes allow the insertion of biologically active molecules into their structures and thus the inspection of fragmented, isolated and well-defined processes of interest [59]. PEDOT, one representative conducting polymer with great environmental stability, conductivity, transparency and biocompatibility [60-71], has been extensively studied due to its easy synthesis, low cost, excellent environmental stability, high charge mobility, low band gap, and high electrical conductivity as well as broad potential applications [72-78]. EDOT oligomers have demonstrated potential applications in biosensors [79-82], organic field effect transistors [83], organic light emitting diodes [84], photovoltaic cells [85], solar cells, memory devices, ion-selective electrodes, microelectrode arrays, fuel cells, actuators, etc. [86-97].

In this paper, we show that the properties of PEDOT films doped with the redox-active dopant, Laccase, change dramatically depending on the potential applied during electropolymerization. Synthesis of new types of polymeric materials with a remarkable electrical conductivity directly connected the polymer synthesis, applications, and electrochemistry. PEDOT improved the adhesion, electroactivity and conductivity of the films. The preparation of conjugated polymers containing additional enzyme functional groups especially metal complexes (Cu^{2+}) and redox-active species (T1) are of concern by polymer scientists.

2. EXPERIMENTAL

2.1. Materials

Laccase (EC1.10.3.2), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 3,4-ethylenedioxythiophene (EDOT), Multi-walled carbon nanotubes (MWCNTs), Cetrimonium bromide (CTAB), Hydroquinone (HQ), Glucose oxidase (GOx) (EC1.1.3.4) were purchased from Sigma–Aldrich (St. Louis, U.S.A.). All other chemicals used were of analytical grade and used without further purification 0.1 M pH 7.0 phosphate buffer solutions (PBS) and pH 1.5 H_2SO_4 solutions were used as supporting electrolyte. Aqueous solutions were prepared using doubly distilled deionized water.

2.2. Apparatus

Cyclic voltammetry (CVs) was performed in an analytical system model CHI-1205A and CHI-1205B potentiostat. A conventional three-electrode cell assembly consisting of an Ag/AgCl reference electrode and a Pt wire counter electrode were used for the electrochemical measurements. The working electrode was Pt electrode (area 0.03 cm^2). In these experiments, all the potentials have been reported versus the Ag/AgCl reference electrode. Electrochemical impedance spectroscopy (EIS)

measurements were performed using an IM6ex Zahner instrument (Kroanch, Germany). The power output measurements system by KEITHLEY 2400.

3. RESULTS AND DISCUSSIONS

3.1. Preparation of PEDOT-laccase/pt modified electrodes

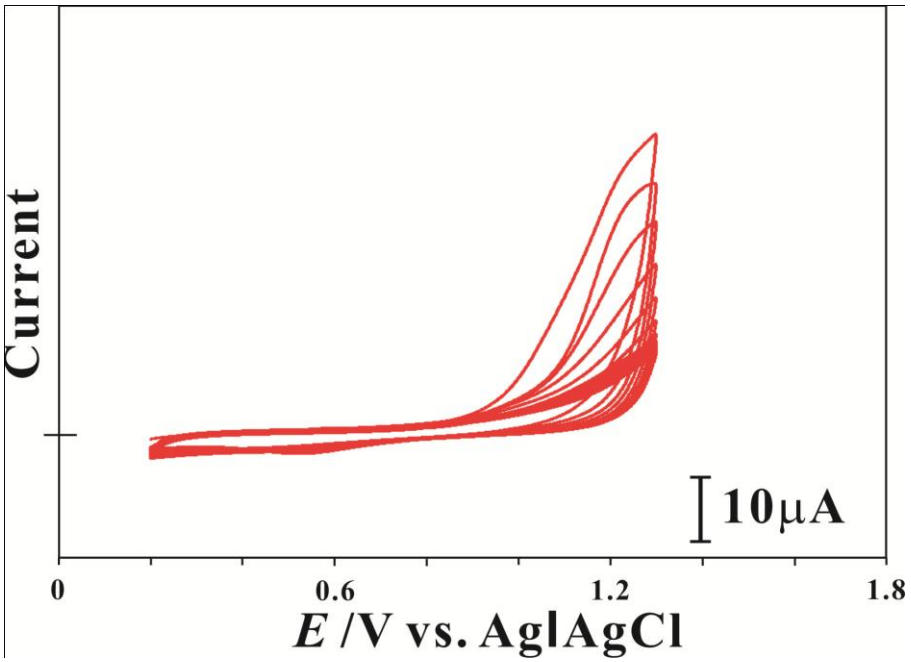

Figure 1. Pt electrode was immersed in pH 1.5 H_2SO_4 solution containing 0.1 mM EDOT and 1 mg/ml laccse from 0.2 V to 1.3 V, scan rate 100 mVs^{-1}.

The common method used for the EDOT polymerization is chemical oxidation of monomer. The generally used oxidants are follows: ammonium persulfate, iron (III) toluenesulfonate, iron (III) chloride, iron (III) sulfate. Major drawbacks of chemical method are its harsh synthetic conditions. Unfortunately, chemical oxidation is carried out under strong acidic conditions using a large amount of oxidizing agents [98-100]. In fact, can be easily prepared by electrochemical polymerisation, which produces a stable deposit on the electrode surface. Fig. 1 shows of modification, Pt electrode was polished with 0.05 μm alumina on Buehler felt pads and then ultrasonically cleaned for about a minute in water. Finally, the electrode was washed thoroughly with double distilled water and dried at room temperature. After that electrode was immersed in pH 1.5 H_2SO_4 solution containing 0.1 mM EDOT and 1 mg/ml laccse from 0.2 V to 1.3 V, scan rate 100 mVs^{-1}. The use of lassase in synthesis of PEDOT is more attractive because the reactions are carried out with lower by-products generation and under milder conditions with a higher degree of kinetic control of the reactions. PEDOT modified electrodes have been already proved to be suitable to oxidise analytes present in laccase, leading to reproducible current/potential responses at 0.509 V and 0.568 V. When an oxidative potential is

applied to a solution containing EDOT, the resulting radical cation electropolymerizes to yield a polymer with positive charges along its backbone. To achieve charge neutrality, anionic species such as a variety of biomolecules including laccase can be attracted and entrapped within the polymer, serving as dopants of PEDOT. Here in, we report a one-pot synthetic approach to an enzyme immobilized electrode based on the electrosynthesis of PEDOT with alginate as a co-dopant of laccase. Our method is easy to perform because the feed ratio of dopants can be controlled easily to tune the properties of the electrode.

3.2. Electrochemical impedance spectra (EIS) of PEDOT-laccase/pt modified electrodes

Electrochemical impedance spectroscopy (EIS), a relatively new and powerful method of characterizing electrochemical properties of materials and their interfaces, is now the method of choice for characterizing interfaces in which the physical and chemical behavior is dependent on several different processes occurring at different rates. In theory, any intrinsic property that influences the conductivity of a enzyme/solution interface can be examined by impedance measurements. Initially applied to the determination of the double-layer capacitance and in ac polarography, they are now applied to the characterization of electrode processes and complex interfaces.

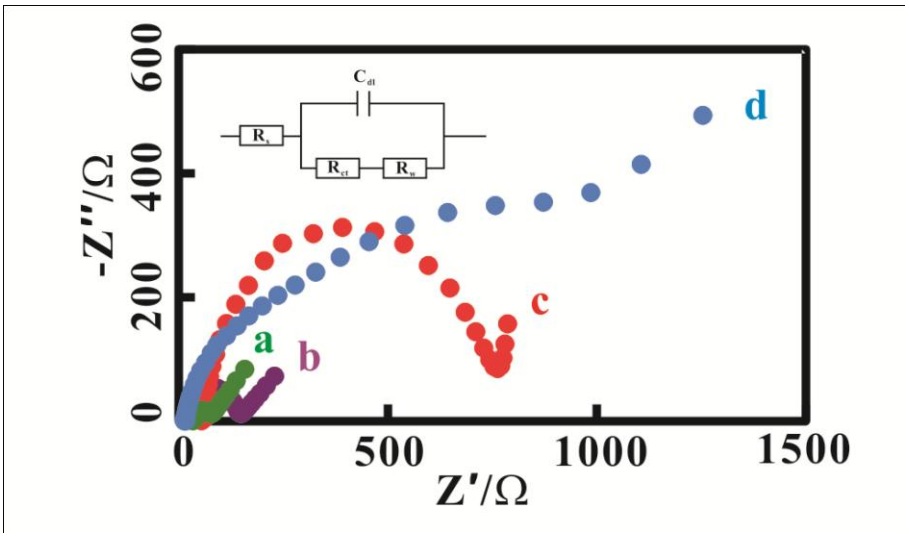

Figure 2. Electrochemical impedance spectroscopy (EIS) for (a) PEDOT/pt, (b) Pt, (c) PEDOT-laccse/pt and (d) laccse/pt modified electrodes in the presence pH 7.0 PBS of equimolar 5 mM $[Fe(CN)_6]^{3-/4-}$. The insert displayed the equivalent circuit (Randles model) was used to fit Nyquist diagrams.

Analysis of the system response contains information about the interface, its structure and reactions taking place there. Fig. 2 shows the results of EIS for different modified electrodes in the presence pH 7.0 PBS of equimolar 5 mM $[Fe(CN)_6]^{3-/4-}$. The Faradaic impedance spectra, presented as Nyquist plots (Z''vs. Z') for the Pt, laccse/pt, PEDOT/pt and PEDOT-laccse/pt modified electrodes. The PEDOT/pt modified electrode exhibited almost a straight line (curve a) with a very small

depressed semicircle arc (R_{et} = 56.11 (Z′/Ω)) represents the characteristics of diffusion limited electron-transfer process on the electrode surface. PEDOT is suitable for the modification electrodes due to their high electronic conductivity for the electron transfer reactions and better electrochemical and chemical stabilities in aqueous solutions. On the same conditions, the bare pt electrode (curve b) shows like a depressed semicircle arc (R_{et} = 133.79 (Z′/ Ω)) clearly indicated the higher electron transfer resistance behavior comparing with PEDOT/pt modified electrode. Laccase-pt (curve d) modified electrode shows highest R_{et} of 873.1 (Z′/ Ω) than others. The increase in the value of electron transfer resistance (R_{et}) due to the coating of enzyme as a barrier on electrode surface. This semicircle arc PEDOT-laccase/pt (curve c) modified electrodes's R_{et} had been found as 744.13 (Z′/ Ω). PEDOT have been considered as organic materials that have characteristic properties such as hydrophobic surfaces, low thermal and electrical conductivities, chemical inertness, and high mechanical durability. The insert displayed the equivalent circuit (Randles model) was used to fit Nyquist diagrams. It constitutes a distributed element which can only be approximated by an infinite series of simple electrical elements. However, a fundamental progress in the polymer science has absolutely changed this prospect.

3.2. Bioelectrocatalysis of oxygen at PEDOT-laccase/pt modified electrodes

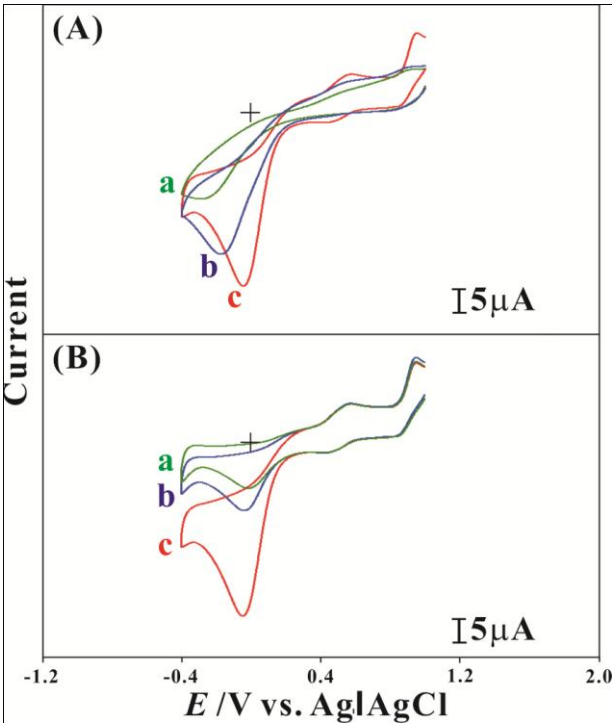

Figure 3. (A) shows PEDOT/pt (a), laccase/pt (b) and PEDOT-laccase/pt (c) modified electrode in 0.1 M PBS (pH 7.0 containing 1 mM ABTS) saturated with oxygen. (B) shows the voltammograms recorded at a PEDOT-laccase/pt modified electrode in 0.1 M PBS (pH 7.0 containing 1 mM ABTS) saturated with nitrogen (curve a), ambient air (curve b) and oxygen (curve c). The corresponding cyclic voltammograms have been obtained at 100 mVs^{-1} scan rate in the potential range of 1.0 to -0.4 V.

Cyclic voltammetry was used to characterize the bioelectrocatalytic activity of laccase electrodes toward the reduction of oxygen. Other attractive materials for immobilizing enzymes include conducting polymers such as PEDOT because they can facilitate the bioelectrocatalysis of enzyme by providing conductive pathways. In order to confer a higher activity to redox oxygen of laccase, the innovative combination of PEDOT was attempted. We previously demonstrated an electrode that can bioelectrocatalyze the conversion of dioxygen to water by immobilizing laccase and its redox mediator, 2,2′-azino-bis(3-ethylbenzothiaxoline-6-sulfonic acid (ABTS). These three kinds of configurations were composed of PEDOT/pt, laccase/pt and PEDOT-laccase/pt modified electrodes. Fig. 3 (A) shows PEDOT/pt (a), laccase/pt (b) and PEDOT-laccase/pt (c) modified electrode in 0.1 M PBS (pH 7.0 containing 1 mM ABTS) saturated with oxygen. PEDOT/pt modified electrode obtained response current 2.01 μA at -0.25 V. Laccase/pt modified electrode appeared higher current and lower overpotential then PEDOT/pt. Electrocatalysis of PEDOT-laccase/pt modified electrode shows current 40.46 μA at -0.03 V. Reduction of oxygen by the biocatalyst yields the responsed laccase redox site. The ABTS mediates redox of laccase.

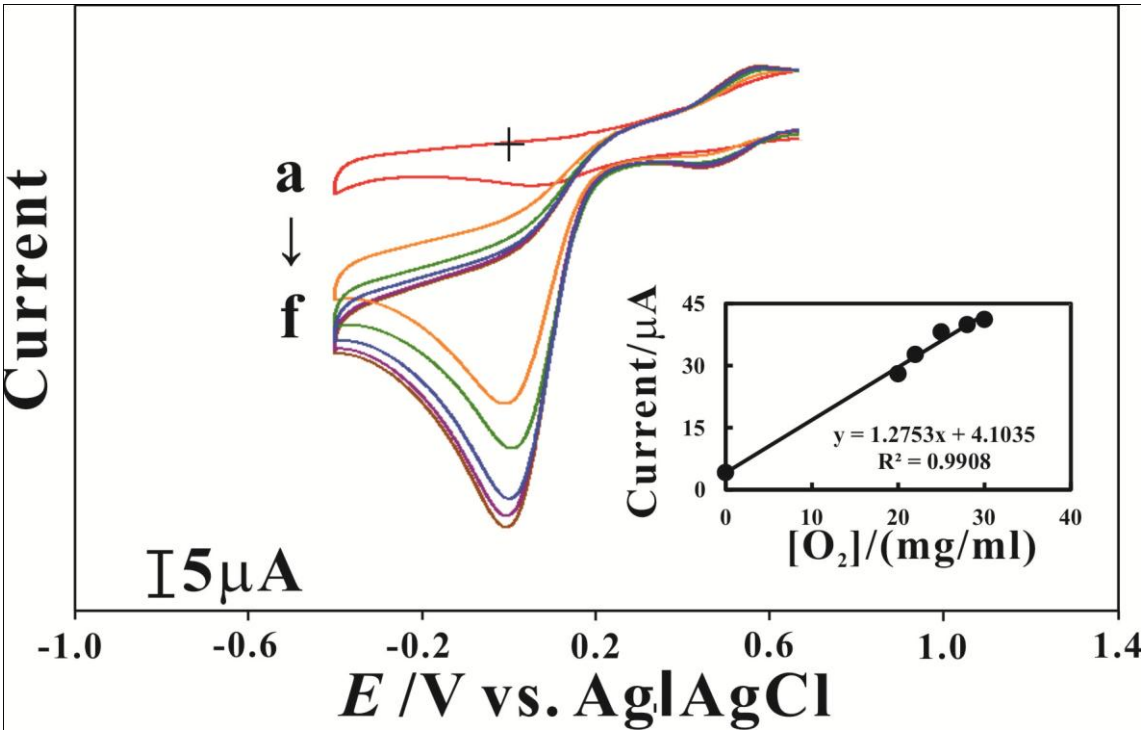

Figure 4. CVs of PEDOT-laccase/pt modified electrode was transferred to pH 7.0 PBS (containing 1 mM ABTS) for the electrocatalysis of oxygen. (a) to (f) upon addition of oxygen. Inset shows a current vs. concentration plot of oxygen at PEDOT-laccase/pt modified electrode.

We suggested that ABTS redox peak near 0.5 V. $ABTS^{2-}$ as one-electron donor for the laccase catalyzed reduction of dioxygen. For instance, Fig. 3 (B) shows the voltammograms recorded at a PEDOT-laccase/pt modified electrode in 0.1 M PBS (pH 7.0 containing 1 mM ABTS) saturated with nitrogen (curve a), ambient air (curve b) and oxygen (curve c). The corresponding cyclic

voltammograms have been obtained at 100 mVs^{-1} scan rate in the potential range of 1.0 to -0.4 V. Under nitrogen atmosphere, as can be seen, there is weak peak in the potential region studied. Curve (b) shows at ambient air (pH 7.0 PBS), we can obtain slight current 16.2 μA. At saturation oxygen conditions, we obtained peak current 40.53 μA, increased current dramatic from laccase reduction reaction and PEDOT. Immobilization of laccase on solid substrates is of widespread interest because the resultant structures are crucial components in biosensors and biofuel cells. Fig. 4 shows the PEDOT-laccase/pt modified electrode was washed carefully in deionized water and transferred to pH 7.0 PBS (containing 1 mM ABTS) for the electrocatalysis of oxygen. Upon addition of oxygen a new growth in the reduction peak of respective analytes have appeared at the current values. An increase in concentration of oxygen, simultaneously produced a linear increase in the reduction peak currents of the oxygen. Inset shows a current vs. concentration plot of oxygen at PEDOT-laccase/pt modified electrode. This seems to indicate that the presence of PEDOT affect the lower potential value and laccase can increase reduction currents. Laccase can electrocatalysis excellently of oxygen. The potential value for the oxygen reduction was close to the redox potential of the laccase used. From all these above results it is clear that PEDOT-laccase/pt modified electrode is more efficient and exhibits enhanced functional properties comparing to that of bare pt alone.

3.3. Optimization of PEDOT-laccase/pt modified electrodes

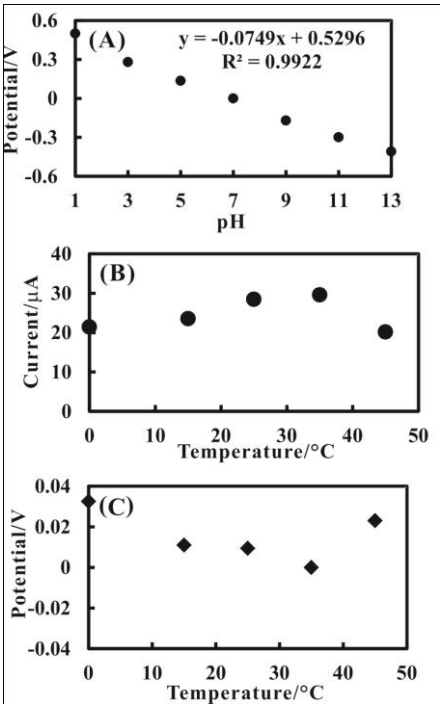

Figure 5. Optimization of PEDOT-laccase/pt modified electrodes in (A) shows the stability plot of PEDOT-laccase/pt modified electrodes in different pH 1.0 to 13 solution. (B) shows PEDOT-laccase/pt modified electrode in pH 7.0 PBS (containing 1 mM ABTS) and saturation oxygen conditions at various temperature from 0 to 45°C. (C) shows various temperature vs. reduction potential.

Laccases are extracellular 55 and 57 kDa acidic enzymes (pI 4 and 3.5), correspondingly, and provide the 4 e^- electroreduction of O_2 to H_2O with a pH optimum of 4 to 4.5. Fig. 5 (A) shows the stability plot of PEDOT-laccase/pt modified electrodes in different pH solution. This result reveals that the modified electrodes exhibits a reasonable stability from pH 1.0 to 13. Here, the good stability of PEDOT-laccase/pt modified electrodes could be attributed to the stability of PEDOT and the high affinity between laccase. The peak potentials shifted to the negative potentials by increasing pH. From different pH, a peak of PEDOT-laccase/pt shows a pH-dependent response, which indicates that protons were involved in the electron-transfer reaction. However, laccase directly immobilized on a solid substrate are prone to inactivation and deterioration of their functions. Therefore, biocompatible modifiers to tailor solid substrates have been proposed. Among the modifiers, PEDOT with promising properties and biocompatibility have attracted considerable attentions for immobilization of bioactive. Fig 5 (B) shows PEDOT-laccase/pt modified electrode in pH 7.0 PBS (containing 1 mM ABTS) and saturation oxygen conditions at various temperature. Typical bell curve, recoded response current from 0 to 45°C. PEDOT-laccase/pt modified electrode revealed highest peak current 29.59 μA at 35 °C. It is unsuited for laccase response at extremely temperature sush as 0 and 45 °C. Fig 5 (C) shows various temperature vs. reduction potential, PEDOT-laccase/pt modified electrode displayed from -0.022 to 0.032 mV. Optimization of PEDOT-laccase/pt modified electrodes is in pH 7.0 PBS and at 35 °C. Ideally, this reaction should proceed close to the $E^{0'}$ for O_2/H_2O reduction, which is advantageous for the development of high-potential biocathodes operating at ambient conditions.

3.4. Biofuel cell performance of PEDOT-laccase/pt modified cathode

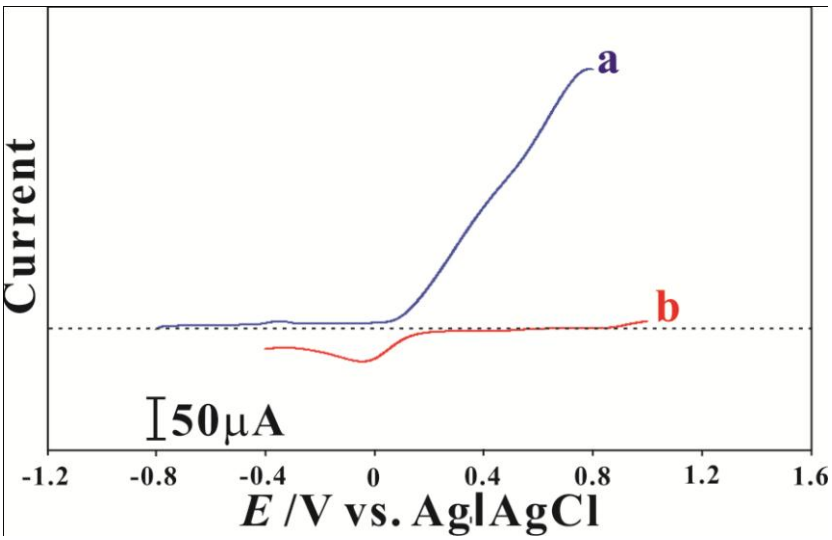

Figure 6. Polarization curves obtained in pH 7.0 PBS for the bioanode GOx/MWCNTs modified GC electrode in a 5 mM glucose solution (curve a) and for the biocathode PEDOT-laccase/pt modified electrode in air-saturated solution (curve b).

We constructed a hybrid biofuel cell with a biocathode that displays an improved stability and increased O_2 reduction currents. To demonstrate the possible potential application in PEDOT-

laccase/pt modified electrodes, prepared GOx/MWCNTs as bioanode [12] and PEDOT-laccase/pt as biocathode had been assembled. Polarization curves shows Fig. 6 that the catalytic oxidation resulting from the glucose oxidation started at about +788 mV (vs. SEC) and the electrocatalytic current on GOx-based bioanode reached a plateau at 612.71 μA cm^{-2}. On the other hand, the electrocatalytic reduction current corresponding to the indirect reduction of dioxygen commenced from +0.03 mV (vs. SEC) and reached a plateau at 42.51 μA cm^{-2}. As model for biofuel cell working under physiological conditions. It displays the relationship between the power density and the cell voltage of the assembled glucose/O_2 biofuel cell in oxygen-saturated 0.1 M PBS (pH 7.0) containing 5 mM glucose. The open circuit potential of the GOx modified electrode is close to the redox potential of the FAD/$FADH_2$ cofactor in the enzyme itself.

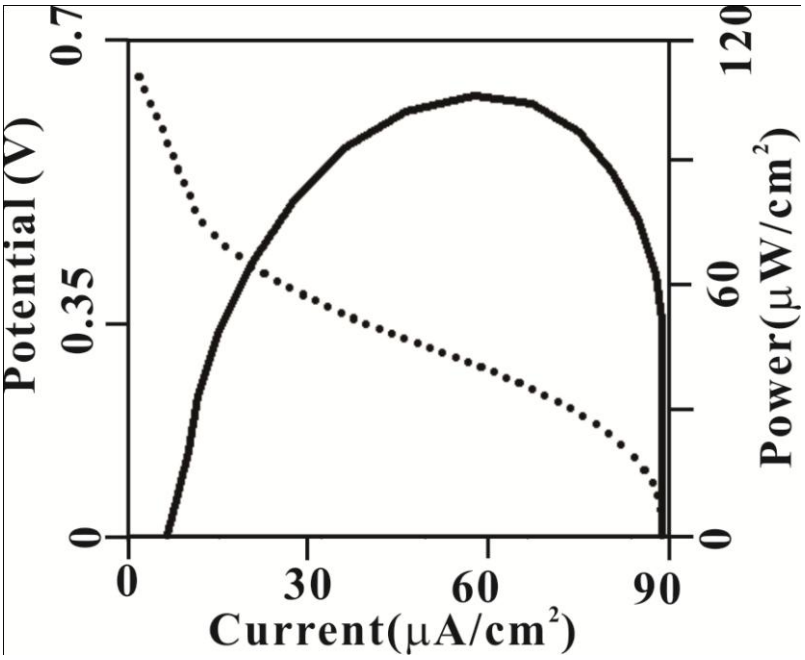

Figure 7. Polarization curve (dashed line) and dependence of the power density on the operating voltage (solid line) for a biofuel cell consisting of GOx/MWCNTs modified electrode as anode in combination with a PEDOT-laccase/pt electrode as cathode. As fuel a 5 mM solution of glucose in PBS (pH 7.0) containing 1 mM ABTS was used.

Fig. 7 shows the influence of the fuel (glucose) and the oxidizer (O_2) on the open circuit voltage of the resulting biofuel cell. According to the Nernst equation, this OCV increase reflects the negative shift of the half-wave potential of mediator due to the enzymatic oxidation of glucose, GOx consuming the oxidized form of mediator at the bioanode. Analysis has shown that the electrocatalytic oxidation current of glucose appears at 0.67 V with a current density of 0.04 μA/cm^2 and reaches 89 μA/cm^2 at 0 V vs. Ag/AgCl. Current density was calculated versus geometric electrode area, giving 0.07 cm^2. In presence of oxygen and laccase, the half-wave potential of ABTS shifts positively due to the enzymatic oxidation of ABTS. As the current is produced, the cell voltage starts to decrease, the cell voltage drops faster and become 0 V at 89 μA/cm^2 of the short circuit current. From the measured

I–V curves, maximum power densities are calculated to be 102 μW/cm^2 at 0.63 V. Although from the practical application point of view, the performance and the stability of the laccse-based glucose/O_2 BFC needs to be further improved, we believe that this initial demonstration can be useful for the development of novel kinds of biosensors and biofuel cells.

4. CONCLUSIONS

In this study, we had revealed the bioelectrocatalytic dioxygen reduction on the PEDOT-laccse/pt modified electrode, respectively. GOx/MWCNTs and PEDOT-laccase/pt as the anodic and cathodic biocatalysts were successfully assembled. The maximum power density of the BFC was 102 μW cm^{-2}. The laccase-based electrodes gave high catalytic currents of O_2 reduction in redox mediators with remarkable operational stability. In conclusion, PEDOT demonstrate great useful potential for the future development of novel kinds of biofuel cells. High sensitivity and stability together with very easy preparation makes PEDOT-laccase electrode as promising candidate for constructing simple electrochemical sensor for oxygen.We report on an alternative concept of biofuel cell functioning based on the unconventional use of enzymes to create a pH difference generating a potential difference between electrodes soaked in ABTS solutions. It is expected that this easy fabrication of enzyme electrodes will paves the way for the development of a new generation of biofuel cells and also will be useful for the development of bioreactors and biosensors.

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