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Enzymatic fuel cells with an oxygen resistant variant of pyranose-2-oxidase as anode biocatalyst

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ABSTRACT

In enzymatic fuel cells (EnFCs), hydrogen peroxide formation is one of the main problems when enzymes, such as, glucose oxidase (GOx) is used due to the conversion of oxygen to hydrogen peroxide in the catalytic reaction. To address this problem, we here report the first demonstration of an EnFC using a variant of pyranose-2-oxidase (P2O-T169G) which has been shown to have low activity towards oxygen. A simple and biocompatible immobilisation approach incorporating multi-walled-carbon nanotubes within ferrocene (Fc)-Nafion film was implemented to construct EnFCs. Successful immobilisation of the enzymes was demonstrated showing 3.2 and 1.7-fold higher current than when P2O-T169G and GOx were used in solution, respectively. P2O-T169G showed 25% higher power output (maximum power density value of 8.45 ± 1.6 μW cm^{-2}) and better stability than GOx in aerated glucose solutions. P2O-T169G maintained > 70% of its initial current whereas GOx lost activity > 90% during the first hour of 12 h operation at 0.15 V (*vs* Ag/Ag^{+}). A different fuel cell configuration using gas-diffusion cathode and carbon paper electrodes were used to improve the power output of the fuel cell to 29.8 ± 6.1 μW cm^{-2}. This study suggests that P2O-T169G with low oxygen activity could be a promising anode biocatalyst for EnFC applications.

1. Introduction

In recent years, development of enzymatic electrodes for bioelectronic applications has attracted many researchers' attention because of their highly efficient catalytic activity for biological reactions (Rasmussen et al., 2016). Enzymatic electrodes are mostly employed in healthcare applications such as biosensors, especially for the detection of glucose (Salek-Maghsoudi et al., 2018), lactate (Rathee et al., 2016), cholesterol (Dey and Raj, 2010) and non-esterified fatty acids (Kang et al., 2014). Enzymatic fuel cells (EnFCs) utilising glucose are another example where enzymatic electrodes have been extensively used to harvest micro-power for implantable and small electronic devices (Yu and Scott, 2010).

Lignocellulosic biomass is the most abundant renewable biological resource available on earth and is a suitable raw material for biofuels and chemicals (Dougherty et al., 2014). EnFCs could be a good alternative to utilise sugars from hydrolysis of lignocellulosic biomass for energy generation, particularly with the safety concerns in aviation travel on Li-battery and alcohol fuel cells used in electronic devices, such as laptops and mobile phones (Kim et al., 2016; Schievano et al., 2016). EnFCs using sugars as fuel for power generation possess advantages with widely available sugar source and non-flammable nature.

Different enzymes and immobilisation approaches for enzyme electrodes have been developed to improve the power output and stability of EnFCs (Rasmussen et al., 2016). One of the most important issues in the development of enzymatic electrodes is the successful and efficient transfer of electrons between enzyme and electrode. A number of different electron transfer mediators have been used to fabricate enzymatic electrodes such as osmium (Os), benzoquinone, poly-vinylferrocene, ferrocene (Fc) and its derivatives (Ivanov et al., 2010). Among all the mediators employed, Fc and its derivatives stand out because of their non-toxicity to human body and their solubility in different solvents such as water and ethanol (Harkness et al., 1993; Stepnicka, 2008).

Several methods have been applied to fabricate Fc integrated enzyme electrodes for biosensors and EnFCs to achieve stable and

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electrochemically active enzyme electrodes (Abdulbari and Basheer, 2017; Saleem et al., 2015; Yang et al., 2003). Coatings of Fc-Nafion films were shown to be promising for the fabrication of long term stable and electrochemically active enzyme electrodes as Nafion is readily permeable to glucose (Dong et al., 1992; Vaillancourt et al., 1999). Although promising results were obtained, fabricated electrodes suffered from several problems such as low electrical conductivity of the films formed, some interferences due to electropolymerization processes and the long-term leaching of both enzyme and mediator. In more recent years, Nafion supported systems was also used for uric acid, dopamine and alcohol biosensors (Chen et al., 2011; Chinnadayyala et al., 2014; Ghosh et al., 2015). However, there is still room for improvement as the properties of these films can be enhanced using novel materials and immobilisation methods.

Carbon nanotubes (CNTs) have been used to enhance the properties of Fc-Nafion films because of their unique properties of biocompatibility and excellent electrical communication it can provide between the enzymes and the electrodes with the integration of electron transfer mediators and polymer matrixes (Dai, 2002; Meredith et al., 2011; Smart et al., 2006; Tran et al., 2011). In particular, non-covalent binding of molecules to the CNTs sidewalls obtaining strong π- π interactions using pyrene and its derivatives provides a wide range of possibilities for enzyme immobilisation (Jönsson-Niedziolka et al., 2010). This approach has been widely used in the field of EnFCs (Güven et al., 2016; Halámková et al., 2012; Krishnan and Armstrong, 2012; MacVittie et al., 2013; Szczupak et al., 2012). High surface area carbon material on the electrode surface can provide successful crosslinking of enzymes and better conductivity using pyrene and/or its derivatives. It has also been reported that dispersions prepared using multi-walled carbon nanotubes (MWCNTs) and Nafion showed promising results for biosensors such as bilirubin and glucose determination (Filik et al., 2015; Mani et al., 2013).

Glucose oxidase (GOx) is one of the most widely used enzyme in EnFCs and has many advantages due to its well-known structure as well as inexpensive, stable and practical use (Heller, 2004; Willner et al., 2009; Wilson and Turner, 1992). It has, however, significant drawbacks including restricted turnover rates for glucose and high turnover rates for oxygen (Zafar et al., 2010). Pyranose-2-oxidase (P2O), on the other hand, is a wood degrading enzyme and has become popular due to its excellent reactivity with alternative electron acceptors for a range of sugar substrates (Odaci et al., 2008; Tasca et al., 2007). As P2O has high specificity toward aldopyranose sugar, it can be applied for detection of D-glucose or 1,5-anhydroglucitol, an analogue of D-glucose found in serum of diabetes patient (Yabuuchi et al., 1989). Unlike GOx, P2O can use both α, and β-D-glucose as substrates, therefore, P2O can generate more redox output from glucose oxidation than GOx which can result higher power outputs in EnFCs (Tasca et al., 2007).

There are several studies toward the immobilisation of P2O for biosensor applications, wherein the co-immobilisation of P2O with peroxidase on a carbon paste electrode was one of the earliest reports (Lidén et al., 1998). A few studies have been reported using P2O with different flexible Os functionalized polymers (Tasca et al., 2007; Timur et al., 2006; Zafar et al., 2010). Although these reports show promising results with Os polymers, there is a concern about their use in implantable devices. Os compounds are toxic and not biocompatible, therefore leaching is a serious concern posing a high risk for long term applications (Yu and Scott, 2010). Other studies demonstrate the use of carbon nanotubes (CNTs) and gold nanoparticle-polyaniline/gelatin nanocomposites with P2O enzyme to fabricate biosensors (Odaci et al., 2008; Ozdemir et al., 2010). According to authors' best of knowledge, there are a couple of studies utilising P2O in EnFCs (Kim et al., 2017; Kwon et al., 2014). Researchers employed P2O and GOx in a fuel cell reaching power densities of 40.7 $\mu W\,cm^{-2}$ using an air-breathing platinum cathode (Kwon et al., 2014). In another one, researchers used enzyme precipitate coating method to immobilise P2O at the anode and platinum at the cathode reaching power density values of 53 $\mu W\,cm^{-2}$ (Kim et al., 2017). In both of the studies, researchers did not present a fully enzymatic system and they used electron mediators in solution for oxidation reactions.

In this study, oxygen insensitive variant of P2O (P2O-169G) was used as anode catalyst in a full enzymatic fuel cell and compared with widely used GOx fuel cell. P2O-169G was constructed using semi-rational protein design and reported not to utilise as much oxygen as their wild type form but still retain the advantages of oxidizing sugars of the wild type enzyme (Pitsawong et al., 2010). Mechanistic details underlying how the active site controls oxygen reactivity of P2O have been elucidated by density functional theory, transient kinetics and site-directly mutagenesis to be involved with proton-coupled electron transfer reaction (Wongnate et al., 2014). P2O also showed good oxidation activity for various sugars, not only specific to glucose (Spadiut et al., 2010). This makes it more attractive for EnFC applications with broader fuel sources.

The behaviour of P2O-T169G enzyme in electrochemical systems can provide important information to develop more efficient and stable electrodes. In a previous study, a comparative study testing the electrochemical behaviour and stability of P2O-T169G and GOx enzymes in oxygen saturated solutions was reported (Sahin et al., 2014). We here report the electrochemical performance of P2O-T169G and GOx when immobilised on Fc-MWCNTs modified carbon screen-printed (SPE) and carbon paper electrodes (CPE) using pyrene crosslinking chemistry. The fuel cell performance of the P2O-T169G as an anode biocatalyst was investigated and compared with the results obtained using GOx. Finally, the P2O-T169G fuel cell was tested in an air-breathing enzyme cathode system. The results suggested P2O-T169G with low oxygen activity is a good candidate for EnFCs.

2. Experimental

2.1. Materials

Chemicals used in this study and the details of the electrodes used are summarised in Table S1. P2O-T169G (prepared using site-directed mutagenesis at position Thr169, 0.2 U/mg) was prepared as reported previously (Pitsawong et al., 2010; Wongnate et al., 2011). MWCNTs (inner diameters of 20–50 nm and outer diameters of 70–200 nm) were obtained from Applied Sciences Inc. (Ohio, USA). Perspex cells used for the experiments were made in house.

2.2. Fabrication of enzyme electrodes

The schematic display of the immobilisation process for SPEs is shown in Fig. 1. Briefly, Fc-Nafion-MWCNTs composite was prepared by mixing 1 mg of MWCNTs in 1 mL of 25 mM Fc containing 1 wt% Nafion solution with 90% ethanol at pH 7 under sonication for 3 h using a similar approach reported before by Dong et al. (1992). A solution of Fc-Nafion-MWCNTs was drop coated onto carbon SPEs in small additions with drying time allowed between each step. Then, the dried electrode was placed in a perspex cell and a preconditioning step of 20 cyclic voltammetry (CV) scans at 50 $mV\,s^{-1}$ between − 0.4 V and 0.4 V (*vs* Ag/Ag^{+}) was applied. After the preconditioning step, the electrode was washed with de-ionised water, dried in an oven at 35 °C for 10 min. Immobilisation of the enzymes were performed using a heterobifunctional cross-linker, 1-Pyrenebutyric acid N-hydroxysuccinimide ester (PBSE, 10 mM in dimethylformamide) for 1 h following by incubating activated electrodes with enzyme solutions for 2 h. Since PBSE has an N-hydroxysuccinimide group attached to the acid, it eliminates the extra carbodiimide + ester step in conventional approaches hence resulting in easier fabrication of enzyme electrodes. The electrodes then were rinsed with de-ionised water and/or phosphate buffer solution (PBS) (0.1 M, pH 7) between each step to remove weakly bonded species and tested in 0.1 M PBS at pH 7 without further treatment. Immobilisation in CPEs were also performed using the same procedure.