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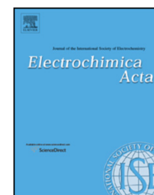

Glucose Oxidase Directly Immobilized onto Highly Porous Gold Electrodes for Sensing and Fuel Cell applications

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ABSTRACT

The successful implementation of redox-enzyme electrodes in biosensors and enzymatic biofuel cells has been the subject of extensive research.

For high sensitivity and high energy-conversion efficiency, the effective electron transfer at the protein-electrode interface has a key role. This is difficult to achieve in the case of glucose oxidase, due to the fact that for this enzyme the redox centre is buried inside the structure, far from any feasible electrode binding sites.

This study reports, a simple and rapid methodology for the direct immobilisation of glucose oxidase into highly porous gold electrodes. When the resulting electrode was tested as glucose sensor, a Michaelis-Menten kinetic trend was observed, with a detection limit of 25 μM. The bioelectrode sensitivity, calculated against the superficial surface area of the bioelectrode, was of 22.7 ± 0.1 μA mM^{-1} cm^{-2}.

This glucose oxidase electrode was also tested as an anode in a glucose/O_2 enzymatic biofuel cell, leading to a peak power density of 6 μW cm^{-2} at a potential of 0.2 V.

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1. Introduction

The functional immobilisation of redox enzymes, such as laccase and glucose oxidase, onto electrode material surfaces is of keen interest for sensors and biofuel cells development.

Enzymes are the most common bioreceptor molecules used in biosensors due to their extremely high specificity that leads to minimal risk of false positive responses. The implementation of enzymes in biofuel cells allows for the development of membrane-less and compartment-less devices, which not only can be easily miniaturised, but can also be used in situations where it is not feasible to separate the fuel and oxidant [1].

For these applications, the achievement of efficient electron transfer between the enzyme active centre and the electrode is critical. Usually a mediated electron transfer (MET) mechanism is required. This might involve the use of small redox active particles and polymers as electron carriers (mediators), such as organic dyes, ferrocene and its derivatives, modified vitamin complexes, and conducting salts [2]. If the mediator is in solution their diffusion to the electrode surface allows for a more rapid electron transfer compared to the direct transfer from the enzyme itself [3]. Alternatively, the mediators can be polymerised directly onto the electrode surface or co-immobilised with the reacting enzymes to further enhance the rate of electron transfer [4,5].

However, the use of redox active electron carriers can have several drawbacks, such as short lifespans, poor biocompatibility, risk of leaching away from the electrode surface, potential toxicity. Consequently, the achievement of a direct electron transfer (DET) process is preferred.

Major advancements have come as far back as the 1980s from examining transition metal rich enzymes, such as laccase (LAC), capable of catalysing the reduction of oxygen to water through DET [6]. This is because LAC contains several copper centres that allow the electrons transport through the enzyme redox centre to the electrode surface. Consequently, the achievement of DET with LAC-immobilised electrodes is now well established [7–9].

In the case of glucose oxidase (GOx), DET is more difficult to achieve, due to the fact that the GOx redox centre is buried inside the enzyme structure, and is far from any feasible electrode binding sites. To achieve efficient electron transfer, the use of GOx has been often combined with mediator compounds, of which ferrocene is the most common [10]. Most of the GOx immobilisation protocols reported, while effective, are usually very expensive, due to the reagents required. These protocols are often very laborious, involving multi-steps in the immobilization procedure that can be sources of experimental errors [11–14]. Moreover the resulting bioelectrodes can be unstable and inefficient with limited opportunity for practical implementations, due to the leaching of

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the mediator implemented and the dependence of the electron transfer process on the capability of the mediator to be rapidly oxidised and reduced [15].

In recent years, some progress has been reported when using nanostructured electrodes, such as carbon nanotubes (CNTs), as electron acceptors. Due to their size and shape, these electrodes are able to intertwine with the enzyme and come in very close proximity to the enzyme FAD centre. Successful DET of GOx immobilised onto CNTs-based electrodes has been reported [16–18]. High surface area microelectrodes are also preferred in the context of an increasing trend towards the miniaturization of bioelectrochemical devices for applications such as implantable healthcare devices. However, the not yet fully addressed potential long-term toxicity of CNTs leads to controversial opinions on the feasibility of using CNTs for *in vivo* or cutaneous applications [19–23].

Nano-porous gold (nPG) electrodes (porous gold electrodes with a pore size distribution limited to the nanometre range) are considered a very promising alternative for the development of new generation bioelectrochemical devices with implantable capability. These non-toxic electrodes have remarkable properties, such as high conductivity, large surface area, three-dimensional open porosity, and biocompatibility [24]. In this context, an even more promising material for the production of high sensitivity biosensors is highly porous gold (hPG). While retaining the morphology observed with nPG, hPG electrodes present large micro-pores that are lined with nano-pores themselves [25]. As a consequence, hPG electrodes have a very wide pore size distribution, leading to extremely large surface areas and hence larger current densities in comparison to conventional nPG.

A new and rapid method of producing hPG electrodes by direct electrodeposition of porous gold films onto gold electrodes was recently reported [26]. These electrodes were characterised by a 3D foam-like structure, with a wide pore size distribution (ranging from 10 nm to 30 μm), and a roughness factor (calculated in terms of electrochemically effective surface area) approximately 10^3 times higher than polished gold. The hPG electrodes showed excellent glucose electrooxidation activity with a detection limit as low as 5 μM [26]. However, the high specificity required for some applications, such as implantable biofuel cell devices where the fuel (e.g. glucose) and the oxidant (e.g. oxygen) are fed to the system as a mixture, demands the implementation of enzymatic electrodes [27].

The large surface area of hPG electrodes and their complex morphology make them an ideal support for enzyme immobilisation at high loadings. This hypothesis is encouraged by the successful production of GOx-immobilised nPG electrodes recently reported. In these cases the GOx immobilisation protocols involved the nPG functionalization with thiol-linker molecules or with conductive polymers, such as poly(3,4-ethylenedioxythiophene), to enhance the electron transfer process [24,28,29].

This study reports for the first time, an efficient, simple, cost-effective, and rapid method for the functional immobilisation of GOx onto hPG surfaces. The immobilisation protocol does not require any electrode pre-treatments with linker molecules or polymers and it is simple to reproduce. In particular, GOx is immobilised onto the hPG surface *via* a one-step electrochemical adsorption process in a phosphate buffer with no additional chemicals. The use of the resulting GOx-immobilised hPG electrode is tested for glucose sensing and for energy harvesting in a glucose/O_2 enzymatic biofuel cell.

2. Experimental

2.1. Materials

Glucose Oxidase (GOx) from *Aspergillus niger*, Laccase (LAC) from *Trametes versicolor*, and all other reagents used were of analytical grade and purchased from Sigma-Aldrich. All aqueous solutions were prepared with reverse osmosis purified water. Gold disk electrodes (2 mm diameter), saturated calomel electrodes (SCE) and platinum counter electrodes were purchased from IJCambria Ltd. Gold disk electrodes were polished between uses with a 0.3 micron aluminium oxide polish (Buehler).

All analytical experiments were performed in phosphate buffered saline (PBS). This was prepared with the following constituents: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4. The pH of this solution was then adjusted to 7 with the drop wise addition of 1 M solutions of HCl and NaOH.

2.2. Deposition of highly Porous Gold (hPG) onto gold electrodes

The hPG was fabricated with a two-step potential process as previously described [26]. Briefly, the gold disk electrodes were immersed in an aqueous electrolyte consisting of 0.1 M $HAuCl_4$ and 1 M NH_4Cl. Gold was then potentiostatically deposited in two steps. Firstly, the system was set at a working potential of -0.7 V (vs. SCE) for 5 s. Afterwards, the potential was stepped down to a value of -4.0 V (vs. SCE) for 15 s.

2.3. Immobilisation of GOx onto hPG electrodes

GOx was electrochemically adsorbed onto the prepared hPG disk electrodes by conducting a total of 6 CV scans between 0.42 V and 0.60 V (vs. SCE) at a scan rate of 1 mV s^{-1}, in a PBS solution containing 0.45 mg ml^{-1} GOx (approximately 8 U ml^{-1} as per activity rating of manufacturer).

As a term of comparison of performance, GOx was also immobilised by absorption. In this case, the hPG electrodes were incubated with the GOx solution in PBS (0.45 mg ml^{-1}) for 1 hour at room temperature, without conducting any CV scans. In both cases, the GOx-hPG electrodes were then thoroughly rinsed three times with PBS to remove any weakly bonded enzyme, and stored in PBS at 4 °C until used.

The amount of GOx immobilised onto the hPG electrodes, was estimated by performing a kinetic assay (provided by Megazyme Ltd.) of the enzyme solution before and after the immobilisation procedure and assuming no enzyme losses during the process.

2.4. Immobilisation of LAC onto hPG electrodes

The surface of the hPG electrodes was modified with a layer of amino-phenyl groups through a simple two-stage process. Firstly, a layer of nitro-phenyl groups was attached by performing two reductive CV scans at 100 mV s^{-1} from 0.6 V to -0.6 V (vs. SCE), in the presence of an acetone electrolyte containing 2 mM p-nitrophenyldiazonium tetrafluoroborate and 100 mM tetrabutylammonium tetrafluoroborate. In the second stage, the nitro groups were exchanged for amino groups by conducting two reductive CV scans at 50 mV s^{-1} from 0.0 V to -1.4 V (vs. SCE), in an aqueous electrolyte containing 10% v/v EtOH and 0.1 M KCl. Afterwards, the electrodes were transferred to a PBS solution containing 80 U ml^{-1} of LAC. The electrodes were kept in this solution overnight at 4 °C in order to facilitate the covalent linkage between the amine functional groups on the gold, and the carboxylate groups on LAC. Finally, the electrodes were rinsed three times with PBS and stored in PBS at 4 °C until required.

2.5. Electrochemical Characterisation

All electrochemical processes were conducted using the Autolab PGSTAT128 N (Metrohm, UK) potentiostat. Cyclic voltammetry (CV) and amperometry tests were performed in a three-electrode electrochemical set-up with a SCE reference electrode and a platinum

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