Biosensors and Bioelectronics 26 (2011) 2383–2389

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Electron transfer from *Proteus vulgaris* to a covalently assembled, single walled carbon nanotube electrode functionalised with osmium bipyridine complex: Application to a whole cell biosensor

Frankie J. Rawson [a,*], David J. Garrett [a], Donal Leech [c], Alison J. Downard [a], Keith H.R. Baronian [b,**]

[a] Department of Chemistry, University of Canterbury, Private Bag 4800, Christchurch, New Zealand
[b] School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand
[c] School of Chemistry, National University of Ireland, Galway, Ireland

ARTICLE INFO

Article history:
Received 6 August 2010
Received in revised form 10 October 2010
Accepted 11 October 2010
Available online 16 October 2010

Keywords:
Proteus vulgaris
Electron transfer
Osmium modified carbon nanotube electrode
Toxicity sensor

ABSTRACT

We report the fabrication and use of electrodes constructed from single walled carbon nanotubes (SWCNTs) chemically assembled on a carbon surface and functionalised with an osmium(II) bipyridine complex (Osbpy). The ability of the electrodes to transduce biologically generated currents from *Proteus vulgaris* has been established. Our investigations show that there are two contributions to the current: one from electroactive species secreted into solution and another from cell redox sites. The modified electrode can be used to monitor cell metabolism, thereby acting as a whole cell biosensor. The biosensor was used in a 1-h assay to investigate the toxicity of ethanol, sodium azide and the antibiotic ampicillin and gave quantitative data that were closely correlated with standard cell plate viability assays. The results provide proof of principle that the whole cell biosensor could be used for high throughput screening of antimicrobial activity. One of the modified electrodes was used for approximately 1000 measurements over four months demonstrating the robustness of the system.

1. Introduction

In recent years there has been a growing interest in "wiring" bacterial cells to electrodes to allow for direct electrochemical communication (Bond et al., 2002; Busalmen et al., 2008; Coman et al., 2009; Gorby et al., 2006; Peng et al., 2010; Reguera et al., 2005; Richter et al., 2008). Most of these authors modified an electrode substrate with a redox species or carbon nanotubes and defined the electrode as including these surface modifications (Coman et al., 2009; Timur et al., 2007; Vostiar et al., 2004). The ability of bacteria to transfer electrons directly to an electrode appears to occur by two possible routes. One mechanism is via membrane redox molecules such as cytochrome complexes of the electron transport chain and trans membrane electron transport systems (tPMET) (Busalmen et al., 2008; Peng et al., 2010). The second mechanism is the direct electron transfer between electrically conducting pili of bacteria to an electrode surface (Reguera et al., 2005).

Previous studies of electron transfer between bacteria and electrodes have included the use of bare carbon electrodes (Tender et al., 2002), a glassy carbon electrode (GCE) modified with multi-walled carbon nanotubes (MWCNTs) (Peng et al., 2010) and a gold electrode modified with osmium redox polymers (Coman et al., 2009). In the latter studies, surface modification of the electrode led to advantageous electrochemical behaviour: higher signals were obtained after modification with osmium redox polymers (Coman et al., 2009) while modification of GCE with MWCNTs improved the reversibility of the electrochemical response. A MWCNT paste electrode, drop-coated with an osmium polymer, was also reported to exhibit greater sensitivity than the analogous surface without MWCNTs (Timur et al., 2007).

The ability of a modified electrode to make contact with cell redox centres obviates the need for soluble mediators. This may simplify the design and use of whole cell biosensors, particularly for on-site applications and for flow systems. Further, the use of soluble mediators in microbial fuel cells (MFCs) is impractical and most 3rd generation MFCs rely on organisms that can transfer electrons without requiring a soluble mediator, i.e. the organism can self-mediate or transfer electrons directly to the electrode (Karthikeyan et al., 2009). However, to accelerate the development of MFCs there is a need for new electrode materials and constructions that will permit the use of organisms that cannot self-mediate or directly transfer electrons to existing electrodes, thus increasing the types of microbial cells that can be used as MFC catalysts.

* Corresponding author. Tel.: +64 3 364 2987x7437.
** Corresponding author. Tel.: +64 3 364 2987x7029; fax: +64 3 364 2590.
E-mail addresses: frankie.rawson@canterbury.ac.nz (F.J. Rawson), keith.baronian@canterbury.ac.nz (K.H.R. Baronian).

0956-5663/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2010.10.016

In this work we have used vertically aligned single walled carbon nanotubes (SWCNTs) covalently functionalised with bipyridine complexes of Os(II) (Osbpy) as electrodes for electron transfer to bacteria. The PPF-SWCNT-Osbpy electrode construct is fully covalently connected: modified SWCNTs are covalently tethered to an aminophenyl layer which is covalently grafted to a glassy carbon-like substrate (pyrolysed photoresist film, PPF) through diazonium cation chemistry (Garrett et al., 2010). The covalent assembly process was adopted because it was expected to lead to a very stable sensing surface.

Confirmation of the utility and stability of the PPF-SWCNT-Osbpy electrode was achieved through the detection of toxicity of three compounds to *Proteus vulgaris*. Use of whole cells, typically bacteria and yeast, is a common approach to rapid toxicity sensing. The simplest method is based on the principle that the rate of cell catabolism is a function of a number of factors such as temperature, pH and substrate supply, and also on the presence of toxic compounds. With all other conditions held constant, the presence of toxins becomes the rate determining factor. These measurements can either give information about the potential toxicity of a sample (D'Souza, 2001; Morris et al., 2005; Rawson et al., 1989; Rogers, 2006), or with genetically engineered organisms (Neufeld et al., 2006) or by detection of specific biomarkers (Pemberton et al., 2010), identification and quantification of a specific toxin can be made. The former approach is well-documented using soluble mediators, but can be simplified by the removal of the requirement for a soluble mediator.

This paper reports the preparation and characterisation of the PPF-SWCNT-Osbpy electrode and describes its use as a whole cell biosensor system for toxicity monitoring.

2. Experimental

2.1. Chemicals

All chemicals were purchased from Sigma–Aldrich unless otherwise stated. A 10 mM stock solution of glucose was prepared in sterile 50 mM pH 7 0.1 M KCl phosphate buffered saline (PBS). Stock solutions (10 mM) of NaN_3 and ampicillin in sterile PBS were prepared daily. Ethanol (96%) was diluted with PBS, as required, for toxicity studies.

2.2. Electrode preparation and cleaning

PPF electrodes (15 mm by 15 mm) were generated on a silicon substrate as described previously (Brooksby and Downard, 2004). PPF samples with a surface resistance of 18–25 $\Omega\,cm^{-1}$ were used for surface modification.

The strategy for preparation of Osbpy-functionalised SWCNTs covalently attached to PPF (PPF-SWCNT-Osbpy) is shown in Fig. 1. PPF samples were modified with aminophenyl (AP) film using a similar reported method (Boland et al., 2008). A 10 mL aliquot of a 10 mM solution of 4-aminobenzylamine in 0.5 M HCl at room temperature was rapidly added to 1 molar equivalent of solid $NaNO_2$ and allowed to react for 3 min. The reaction solution was then poured into the electrochemical cell and the 4-aminobenzene diazonium cation was electrografted to the PPF scanning from 0.2 V to −0.6 V followed by fixed potential deposition at −0.6 V for 2 min and finally a further scan from 0.2 V to −0.6 V to confirm passivation (and hence grafting) of the electrode.

SWCNTs (Carbon Nanotechnologies Incorporated) were cut and functionalised with carboxylic groups by sonication for 10 h in a 3:1 mixture of concentrated H_2SO_4 and HNO_3. 25 mg of uncut SWCNTs were added to 27 mL of acid. The bath was cooled by adding ice approximately every 20 min. Following sonication, the contents were poured into 500 mL of distilled water and left to settle overnight. The SWCNTs were then filtered through a 0.22 μm hydrophilic PVDF filter (Millipore) under suction, with washing until the rinse water was close to pH 7. Once dried the SWCNT mats were peeled off the filter discs. Suspensions of cut SWCNTs were prepared by sonication of dried SWCNTs mats in dimethyl sulfoxide (DMSO). SWCNTs were coupled to AP-modified PPF samples by submerging modified PPF samples in a 0.2 mg mL^{-1} DMSO suspension of cut SWCNTs containing of 0.5 mg mL^{-1} dicyclohexyl carbodiimide (DCC). The reactants were sonicated for 30 min and then heated to 65 °C for 24 h in a closed cell. After preparation, PPF-SWCNT electrodes were sonicated in acetone for 2 min and isopropyl alcohol for 10 s and finally rinsed in Milli-Q water. The electrodes were dried with nitrogen gas between each washing step.

Osmium(II)bis-2,2-bypyridine(p-aminomethylpyridine)chlorido hexafluorophosphate was prepared using a modified literature procedure (Jenkins et al., 2009; Zakeeruddin et al., 1992). 100 mg of NH_4OsCl_6 (0.23 mmol) was added to 10 mL of ethylene glycol and stirred for 15 min under nitrogen. 2.1 molar equivalents of 2,2′-bipyrdine (78 mg) was added and the solution refluxed under nitrogen for 1 h. The solution was cooled to room temperature and 10 mL of saturated $Na_2S_2O_4$ was added to ensure full reduction of the Os(III) to Os(II) centre. The solution was stirred at 0 °C for 1 h and the product, $Os(bpy)_2Cl_2$,was collected by filtration. 100 mg of $Os(bpy)_2Cl_2$ (0.174 mM) was added to 10 mL ethylene glycol and stirred for 10 min. 1.1 molar equivalents of 4-aminomethylpyradine (20.7 mg) was added and the solution refluxed for 1 h. After cooling, excess (approximately 100 mg) NH_4PF_6 in 10 mL water was added, the solution was stirred at 0 °C for 1 h and the product, $Os(bpy)_2Cl$(4-aminomethyl pyridine)·PF_6 was collected by filtration.

$Os(bpy)_2Cl$(4-aminomethyl pyridine)$^+$ was coupled to the carboxylic acid-terminated tips of immobilised SWCNTs by first submerging the SWCNT-modified PPF samples in a 40 mM aqueous solution of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDCI) and 10 mM N-hydroxysuccinamide (NHS) solution for 1 h and then transferring the samples to a 5 mM solution of the complex dissolved in pH 6 phosphate buffer and ethanol (7:3). The electrodes were allowed to react for 24 h before being removed and washed with Milli-Q water.

After each use, cells if present, were discarded and the electrodes were immersed in 70% ethanol for 1 min to kill any remaining cells and then rinsed in distilled water, air dried and stored at room temperature.

2.3. Cell culture

P. vulgaris (NTC4175) strain number 6 was maintained on tryptic soy agar at 4 °C. Bacterial cultures were grown in 50 mL of organic media 79 broth (10 g peptone, 10 g glucose, 6 g NaCl, 2 g of pancreatic of digest, 2 g of yeast extract per litre). Media were sterilised before use. A pre-culture was incubated in a shake flask at 37 °C, 180 rpm for 5 h and then 10 mL was transferred to 50 mL of the same medium and cultured for a further 18 h at 37 °C. Cells were harvested in a centrifuge at 4500 × *g* and washed twice in 25 mL of sterile 0.05 M pH 7 PBS. The optical density of the cell suspension was adjusted to give an OD_{600} of 10 using an LKB Novaspec II spectrophotometer. Cells were used over a 5 h period and kept at 5 °C between each assay.

2.4. Electrochemical measurements

Electrochemical measurements were made using an eDAQ potentiostat and PowerLab 2/20 with eDAQ EChem data acquisition software. All measurements were made in a Faraday cage

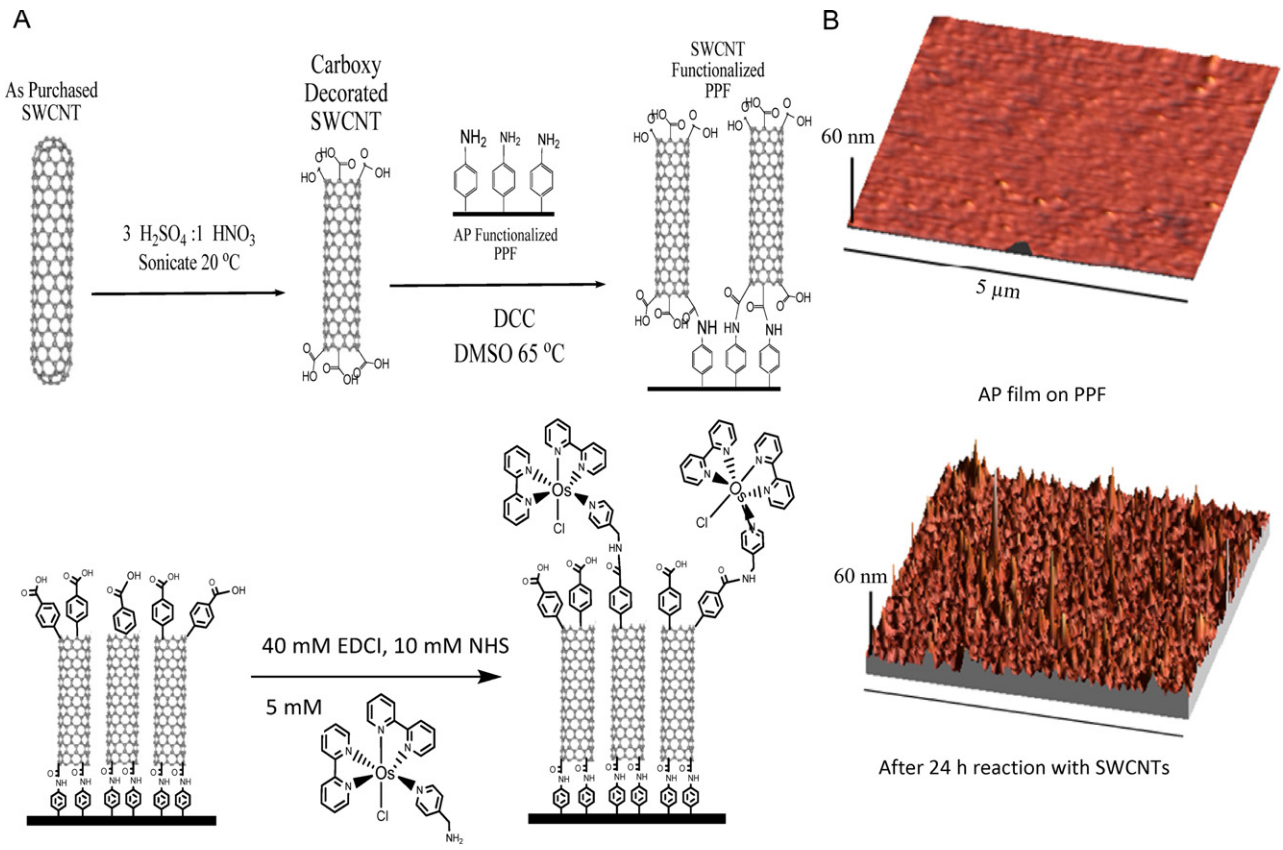

Fig. 1. (A) Schematic diagram of the fabrication procedure for PPF-SWCNT-Osbpy electrodes and (B) AFM images of AP- and AP-SWCNT modified PPF. Adapted from Garrett et al. (2010).

at 37 °C. The working electrode was PPF-SWCNT-Osbpy, bare PPF or PPF modified with chemically assembled SWCNTs. The working electrode was positioned horizontally in the bottom of the electrochemical cell. A viton o-ring (8 mm diameter) defined a working area of 0.50 cm^2. The reference and auxiliary electrodes were Ag/AgCl and Pt, respectively.

Unless otherwise stated, linear sweep voltammograms (LSVs) of *P. vulgaris* were performed at a scan rate of 2 mV s^{-1} with an initial potential of 0.0 V and a final potential of 0.6 V. Working solutions contained cells at an OD_{600} of 1.0 and 1 mM glucose in 50 mM PBS pH 7. Unless otherwise stated, cells were incubated in the electrochemical cell (which included the working electrode) at 37 °C for 1 h prior to electrochemical measurement. The experimental control was a solution of 50 mM PBS pH 7 and 1 mM glucose.

For electrochemical investigation of cell pellets and supernatants, cells were harvested and washed as described in Section 2.3, and the cell pellet was placed on the PPF-SWCNT-Osbpy electrode. LSV was performed as described in above, but without an incubation period. The cell pellet was then transferred to a 1.5 mL eppendorf tube and centrifuged at 12,000 rpm for 12 min. The supernatant was removed and transferred to the electrochemical cell for investigation by LSV. The cell pellet was re-suspended in 1 mL of buffer containing 1 mM glucose and incubated at room temperature for 5 h. This was then centrifuged at 12,000 rpm for 10 min and the supernatant placed in the electrochemical cell and analysed by LSV.

2.5. Toxicity monitoring

Toxin assay solutions (10 mL) contained 1 mM glucose and *P. vulgaris* cells at an $OD_{600\,nm}$ of 1 and added toxins: ethanol with final concentrations of 12.5%, 25%, 50%, and 75%; sodium azide with final concentrations of 0.125, 0.25, 0.5; and 1.0 mM and ampicillin with final concentrations of 0.5, 1, 2 and 4 mM. The assay control was a 10 mL solution of 1 mM glucose and cells at an $OD_{600\,nm}$ of 1. The assay solution was added to the electrochemical cell and placed in a 37 °C incubator for 1 h prior to analysis by LSV. LSV was also performed on blank solutions which contained 1 mM glucose and the corresponding concentration of toxin. Three LSVs were obtained for each solution.

At the end of the voltammetric analysis the assay solution was agitated to ensure an even distribution of the cells before preparing dilutions of 1×10^3 and 1×10^6. A 100 μL aliquot of each dilution was spread on a tryptic soy agar plate. Spread plates were prepared in triplicate and were incubated at 37 °C for a minimum of 16 h before counting.

3. Results and discussion

3.1. Electrochemical characterisation of PPF-SWCNT-Osbpy and cellular electrochemistry

The procedure for preparing PPF electrodes modified with covalently-coupled SWCNTs and subsequent functionalisation with an Os(II) bipyridine complex (PPF-SWCNT-Osbpy) is outlined in Fig. 1. Preparation of the SWCNT-modified surface has been described in detail previously and the surfaces have been fully characterised by microscopy and electrochemical measurements (Garrett et al., 2010). Attachment of the Os(II) bipyridine complex via amide bond formation between an amine-substituted pyridine ligand and carboxylate groups on the SWCNTs followed the general procedure previously established for coupling the complex to carboxylate-terminated surface films (Boland et al., 2008). Cyclic

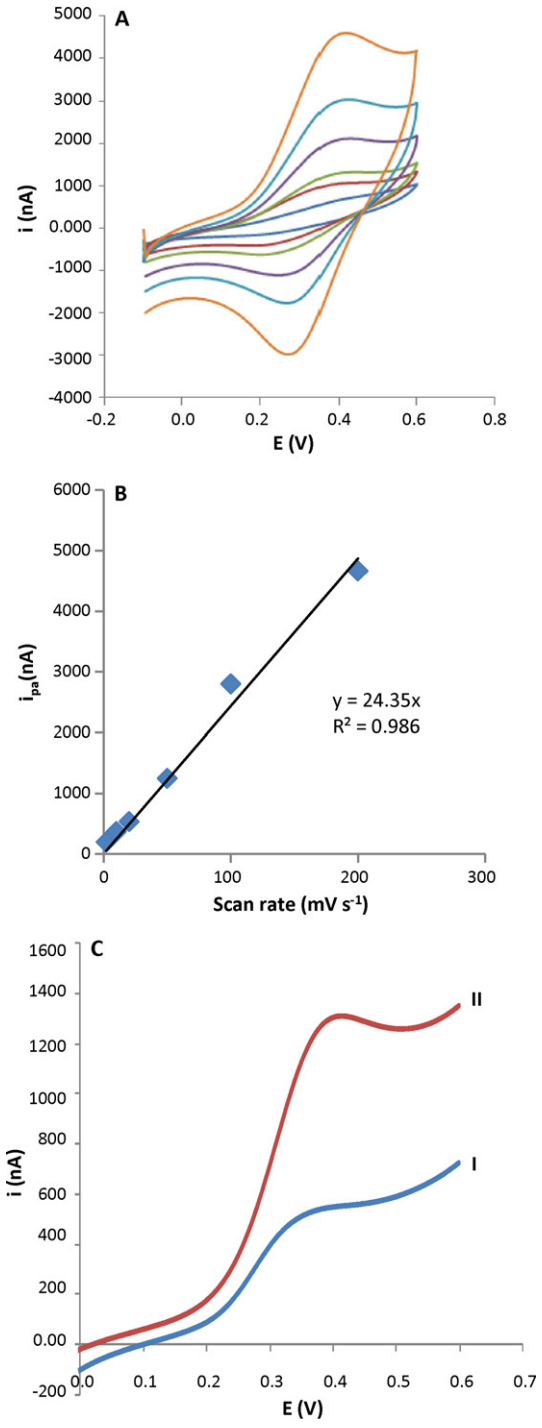

Fig. 2. (A) CVs obtained in solutions of 50 mM PBS, pH 7 at a freshly prepared PPF-SWCNT-Osbpy electrode. CVs were obtained at scan rates of 2, 10, 20, 50, 100, and 200 mV s^{-1}. (B) Plot of oxidation peak currents versus scan rate for the CVs in (A). (C) Typical LSVs obtained in the absence (I) and presence (II) of *P. vulgaris* cells which were incubated for 1 h on the PPF-SWCNT-Osbpy electrode prior to obtaining the LSV.

voltammetry of the surfaces in 50 mM PBS, pH 7 containing 1 mM glucose (Fig. 2A) shows a chemically reversible couple with an $E_{1/2} \sim 0.274$ V vs Ag/AgCl. This $E_{1/2}$ is very close to that reported for the $Os^{2+/3+}$ couple of the similar complex covalently attached to a glassy carbon electrode surface grafted with carboxyphenyl groups ($E^{\circ} \sim 0.268$ V vs Ag/AgCl), consistent with immobilisation of the complex (Boland et al., 2008). Further evidence for surface immobilisation was obtained by examining the relationship between oxidation peak currents and scan rate. Fig. 2B shows there is a linear relationship for scan rates between 2 and 200 mV s^{-1}, as expected for a surface-confined redox process.

Table 1
Oxidation peak currents at a PPF-SWCNT-Osbpy electrode obtained from LSVs of a cell pellet and supernatant without prior incubation of cells, and supernatant after a 5 h incubation.

Conditions	i_{pa} (nA)	Blank subtracted (nA)
PBS blank	292	
Cell pellet	768	476
Supernatant	347	55
Supernatant after 5 h	500	308

The ability of the PPF-SWCNT-Osbpy electrode to interact with cellular redox processes was examined by LSV (scan rate = 2 mV s^{-1}) in the absence and presence of *P. vulgaris* cells (Fig. 2C). A LSV was obtained in the absence of cells (scan I) and then cells were added to give an $OD_{600\,nm}$ of 1. After incubation at 37 °C for 1 h, the LSV was repeated (scan II). Addition of cells gives an increased current for oxidation of the complex, indicating that electrochemically generated Os^{3+} centres are reduced by the cells leading to catalytic enhancement of the signal. The mechanism for this is an EC' process (Kissinger and Heineman, 1984): scanning in a positive direction electrochemically oxidises the Os^{2+} to Os^{3+}, the cells then chemically reduce Os^{3+} back to Os^{2+}, presumably via membrane bound redox sites, leading to redox cycling of osmium centres and the observed increased currents (Fig. 2). Other workers have shown that such catalytic enhancement depends on cell metabolic rate and thus can be used to monitor this rate (Timur et al., 2007). This forms the basis of the toxicity sensor described in the next section.

When the experiment was repeated using bare PPF and PPF modified with non-functionalised SWCNTs, no oxidation peaks were obtained in the presence or absence of cells, confirming that the immobilised Osbpy is essential for mediating electron transfer from the *P. vulgaris*.

There are many reports of 'wiring' of cells to electrodes for electrochemical communication (Alferov et al., 2009; Coman et al., 2009; Timur et al., 2007; Vostiar et al., 2004). Wiring implies that the observed current enhancement in the presence of cells comes from redox centres incorporated in the cell membrane rather than from soluble redox-active cell exudates. To investigate the origin of the cell-related current enhancement observed for *P. vulgaris* at the PPF-SWCNT-Osbpy electrode (Fig. 2C), LSVs were obtained for a cell pellet (prepared by centrifugation) and for the supernatant collected after microcentrifugation of the pellet (Section 2.4). After electrochemical measurement, the cell pellet was re-suspended in 1 mL of 1 mM glucose in PBS and incubated for 5 h and analysis of the supernatant was repeated. The magnitudes of the oxidation peak current of all LSVs were measured by extrapolating the baselines and measuring the peak heights, giving the results shown in Table 1.

The data in Table 1 reveal that current is generated both by the cell pellet (which incorporates cells and supernatant) and the supernatant alone. This confirms that there is direct wiring of the PPF-SWCNT-Osbpy electrode to membrane redox sites of *P. vulgaris* that are in contact with the electrode. The blank-corrected current for supernatant collected from the cell pellet without a prior incubation period is very small, but after 5 h incubation of cells and extraction of the supernatant, the current is significantly larger, consistent with accumulation of a species secreted from the cells. Hence these results establish that in the presence of *P. vulgaris*, current is generated both from cells wired to the electrode and also from a solution species released by the cells.

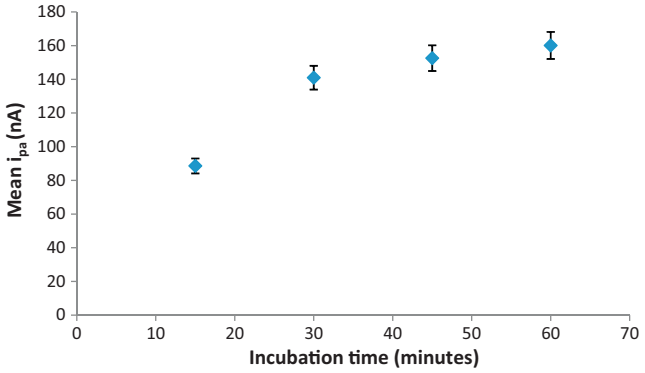

Fig. 3. Mean LSV anodic peak currents versus incubation time for solutions containing *P. vulgaris* at an $OD_{600\,nm}$ of 1 in the presence of 1 mM glucose. Incubation was at 37 °C in the electrochemical cell. Error bars represent ±1 SE of the mean ($n = 3$).

The finding that the biologically generated oxidation currents are largely due to wired cells led to an investigation of the optimum time for incubating *P. vulgaris* on the surface of the electrode, prior to obtaining LSVs. All measurements were carried out using cells stored at 5 °C for 90 and 165 min after harvesting, as well as cells used immediately, and the mean currents obtained at each incubation time are plotted in Fig. 3. Two important points are highlighted by the data. First, for cells with an $OD_{600\,nm}$ of 1 in the presence of 1 mM glucose, the peak currents increase with incubation time (at 37 °C) up to 45 min and are approximately constant over a longer period. Thus it appears that the cells take approximately 45 min to settle and form the maximum number of connections with the electrode and produce the maximum current. Second, the constant peak currents obtained after 45 and 60 min incubation times establish that cell metabolism is constant over this time period and hence that the concentration of glucose is not limiting cell metabolism over a 1 h incubation. Thus all further measurements were made with *P. vulgaris* at an $OD_{600\,nm}$ of 1, with 1 h incubation in the electrochemical cell at 37 °C in the presence of 1 mM glucose. It should be noted however that there are significant changes in response even after 15 min incubation. Hence shorter incubation times could be used for biosensing applications, giving much reduced assay times.

The long term stability of the PPF-SWCNT-Osbpy electrodes was established by comparing the mean anodic peak currents of LSVs obtained at the same electrode in blank solutions (50 mM PBS with added 1 mM glucose) over 96 days. During this time period, the electrode was used for approximately 1000 measurements, the majority in the presence of *P. vulgaris*. Fig. 4 shows that before use in cell solution (0 days datum point) the mean anodic peak current

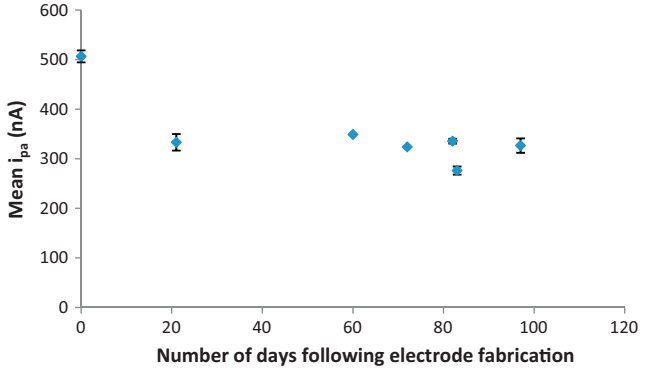

Fig. 4. Magnitude of the mean anodic peak currents obtained from LSVs obtained at a PPF-SWCNT-Osbpy electrode in a solution of 50 mM PBS at pH 7 with 1 mM glucose at a scan rate of 2 mV s^{-1} over a 96-day time period. Error bars represent ±1 SE of the mean ($n = 3$ except for days 60 and 72 for which $n = 1$).

was 507 nA with a coefficient of variation (CoV, SD/mean × 100) of 6.0%. There was a steady decrease in response after use in the presence of *P. vulgaris*, but after 20 days and approximately 50 uses, the response became constant, with a CoV of 9.3%. We assume that use of the electrode in the cell solution either results in adsorption of a layer of electroinactive cell-derived species that decrease**s** the response or alternatively, that surface active cell-derived species remove non-covalently coupled SWCNTs or Os(II) bipyridine complex from the surface. The latter proposal is supported by the results of Boland et al. (2008) who investigated the stability of modified electrodes prepared by reaction of the same osmium complex with an acid-terminating tether layer grafted to GC. Those workers noted an initial decrease in response of the osmium complex on repeated potential cycling which they attributed to the removal of non-covalently bound material (Boland et al., 2008). Importantly, in the present work, the surface attained after 20 days use is highly stable. This stability is attributed to the mode of electrode modification: the AP tether layer is covalently grafted to the carbon substrate with formation of C–C bonds. SWCNTs are coupled to the tether layer through hydrolytically stable amide bonds and similarly the Os(II) bipyridine complex is attached to the SWCNTs via amide bonds. Interestingly however, even after 20 days use, the biologically derived current was observed to decrease slowly with increased number of uses in cell solutions. This phenomenon is currently unexplained but we tentatively suggest that with increased use, cell debris on the surface of the electrode forms a physical barrier making it more difficult for cell wiring. It should also be noted that repeat preparations of PPF-SWCNT-Osbpy electrodes gave surfaces with somewhat different currents for the $Os^{2+/3+}$ couple, but all surfaces gave reliable measurement of biologically derived currents.

3.2. Application of PPF-SWCNT-Osbpy electrodes in toxicity testing

The ease of use and excellent stability of PPF-SWCNT-Osbpy electrodes are ideal properties for sensors for rapid routine assays such as toxicity testing. To demonstrate this application, three toxins with different mechanisms of toxicity were selected: ethanol (cell membrane disruptor), azide (catabolism disruptor) and ampicillin (cell wall synthesis inhibitor). *P. vulgaris* at an $OD_{600\,nm}$ of 1 was incubated for 1 h in the electrochemical cell in the presence of 1 mM glucose and varying concentrations of each toxin. LSVs were recorded at the end of this period giving the mean, blank-subtracted anodic peak currents shown in Fig. 5 (A), (C) and (E). (For these experiments, the blank currents were obtained from LSVs recorded in the presence of 1 mM glucose and the toxin at the appropriate concentration.) To corroborate the electrochemical toxicity results, cell viability counts were carried out in parallel giving the data shown in Fig. 5 (B), (D) and (F). For all toxins, the electrochemical assay gives very similar results to the cell viability counts. For example, in the presence of 12.5% ethanol, the electrochemical signal (Fig. 5A) decreases by 70% while the number of viable cells decreases by 72% (Fig. 5D). This indicates that the PPF-SWCNT-Osbpy electrode can accurately monitor the decrease in biologically generated currents revealing the inhibitory effects of ethanol on the bacterial cells. Ethanol is toxic because it interacts with biological membranes, resulting in decreased membrane integrity which causes an increased flux of protons into the cell, leading to the dissipation of the proton motive force and a decrease in ATP production, resulting in death of the cell (Sikkema et al., 1995).

It is interesting to note that at ethanol concentrations of 25%, 50% and 70%, small but easily measurable signals are obtained using the PPF-SWCNT-Osbpy electrode (Fig. 5A), but plate counts showed that the mean number of viable cells was 4.33×10^5, 1.33×10^5,

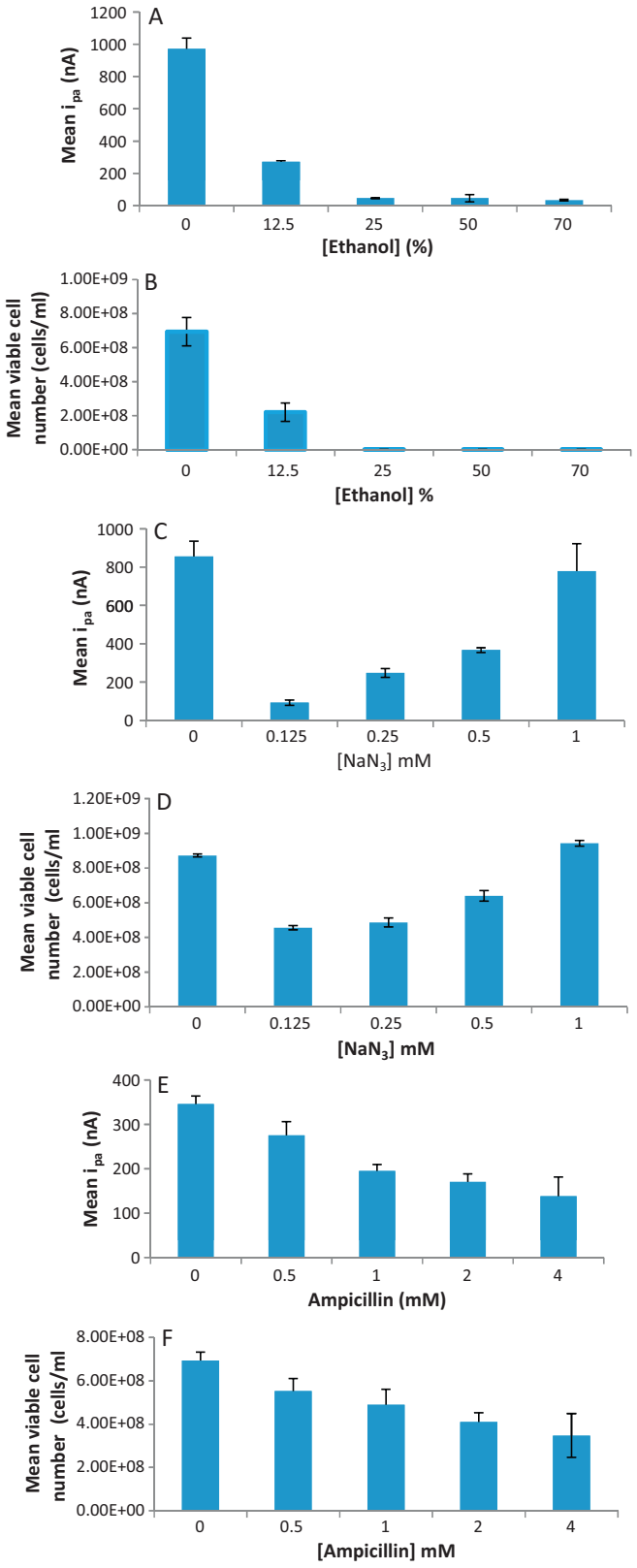

Fig. 5. Plots of mean peak currents (A, C, E) obtained from LSVs of *P. vulgaris*, and viable cell numbers calculated from plate viability counts (B, D, F) exposed to varying concentrations of ethanol (A, B), sodium azide (C, D) and ampicillin (E, F). LSV peak currents have been blank subtracted using LVSs obtained for corresponding solutions in the absence of cells. Error bars represent ±1 SE of the mean ($n = 3$).

6.67×10^5 cells/mL, respectively (Fig. 5B). This indicates that the cells are respiring but at these concentrations they are unable to replicate. Similar behaviour has been reported for *Saccharomyces cerevisiae* cells held at 45 °C and then subcultured on agar plates. Metabolism was detected in the cells at 45 °C but they could not replicate on the agar plate (Kiran Sree et al., 2000; Schaetzle et al., 2008; Walker and Walker, 2006).

Sodium azide is a well known toxin for gram negative bacteria (Lichstein and Soule, 1944) and is used as a positive control in the Ames bacterial mutagenicity assay (McCann et al., 1975). Surprisingly, the electrochemical assay showed that lower levels of sodium azide are more toxic than higher levels (Fig. 5B); this finding was confirmed by cell viability counts (Fig. 5E). These results are in contrast to those reported by Lichstein and Soule (1944) who found a positive correlation between increasing dose of sodium azide and inhibition of growth for *P. vulgaris*. It appears that in the strain of *P. vulgaris* used in the present work, higher doses of sodium azide increase the transcription and translation of genes which limit the toxicity. Sodium azide is known to inhibit protein translocation and one mechanism of resistance has been shown to be a mutation in the protein translocation gene. A mutation to a nucleotide base in the gene that codes for this protein would result in insertion of a different amino acid. The resulting conformational change to the protein may prevent sodium azide from binding allowing translocation to continue. Such a mechanism may explain why sodium azide is less toxic at the higher doses (Oliver et al., 1990).

The final example demonstrating the reliability of the electrode used ampicillin, a bactericide which inhibits cell growth by inhibiting the production of the cell wall (Kaldalu et al., 2004). The toxicity of ampicillin, revealed by the electrochemical assay, shows excellent agreement with the plate viability results (Fig. 5C and F). It should be emphasised that for all of these examples the electrochemical biosensor assay was undertaken with a 1 h incubation whereas the plate counts required at least 16 h incubation. Furthermore, as noted above, a shorter incubation time could be used with the electrochemical assay making this a suitable method for rapid screening.

3.3. Conclusions

This work demonstrates that PPF electrodes that are modified with chemically assembled SWCNTs and functionalised with an Os(II) bipyridine complex can accept electrons from bacterial cell surface redox sites. In addition, a soluble electroactive cell exudate is also shown contribute to the measured current. Work is underway to characterise and identify this species. A key advantage of the sensing surface is its long-term stability, which is attributed to the fully covalent incorporation of components onto the surface (over four months use and approximately 1000 measurements). Incorporation of the Os(II) bipyridine complex on the sensing surface removes the need for a soluble mediator and simplifies use of the electrochemical approach.

The ability of the electrode to accurately report cell metabolic rate has been applied to toxicity sensing, giving results that show good agreement with those obtained from plate viability counts. This electrode will be useful in a wide range of applications including environmental toxicity assessment, sensing applications such as antibiotic screening and biochemical oxygen demand (BOD). This electrode will also permit the use of organisms that cannot self-mediate, thus increasing the types of microbial cells that can be used in microbial fuel cells.

Acknowledgements

This research was funded by the Royal Society of New Zealand, Marsden Fund (contract UOC 0605).

D.J.G. thanks the New Zealand Tertiary Education Commission for a Top Achiever Doctoral Scholarship.

References

Alferov, S., Coman, V., Gustavsson, T., Reshetilov, A., von Wachenfeldt, C., Hägerhäll, C., Gorton, L., 2009. Electrochim. Acta 54 (22), 4979–4984.

Boland, S., Barrieère, F., Leech, D., 2008. Langmuir 24 (12), 6351–6358.

Bond, D.R., Holmes, D.E., Tender, L.M., Lovley, D.R., 2002. Science 295 (5554), 483–485.

Brooksby, P.A., Downard, A.J., 2004. Langmuir 20 (12), 5038–5045.

Busalmen, Juan, P., Esteve-Núñez, A., Berná, A., Feliu, J.M., 2008. Angew. Chem., Int. Ed. Engl. 47 (26), 4874–4877.

Coman, V., Gustavsson, T., Finkelsteinas, A., von Wachenfeldt, C., Haägerhaäll, C., Gorton, L., 2009. J. Am. Chem. Soc. 131 (44), 16171–16176.

D'Souza, S.F., 2001. Biosens. Bioelectron. 16 (6), 337–353.

Garrett, D.J., Flavel, B.S., Shapter, J.G., Baronian, K.H.R., Downard, A.J., 2010. Langmuir 26 (3), 1848–1854.

Gorby, Y.A., Yanina, S., McLean, J.S., Rosso, K.M., Moyles, D., Dohnalkova, A., Beveridge, T.J., Chang, I.S., Kim, B.H., Kim, K.S., Culley, D.E., Reed, S.B., Romine, M.F., Saffarini, D.A., Hill, E.A., Shi, L., Elias, D.A., Kennedy, D.W., Pinchuk, G., Watanabe, K., Ishii, S.I., Logan, B., Nealson, K.H., Fredrickson, J.K., 2006. Proc. Natl. Acad. Sci. U.S.A. 103 (30), 11358–11363.

Kissinger, P.T., Heineman, W.R., 1984. Laboratory Techniques in Electroanalytical Chemistry. Marcel Dekker, New York.

Jenkins, P.A., Boland, S., Kavanagh, P., Leech, D., 2009. Bioelectrochemistry 76 (1–2), 162–168.

Kaldalu, N., Mei, R., Lewis, K., 2004. Antimicrob. Agents Chemother. 48 (3), 890–896.

Karthikeyan, R., Sathish kumar, K., Murugesan, M., Berchmans, S., Yegnaraman, V., 2009. Environ. Sci. Technol. 43 (22), 8684–8689.

Kiran Sree, N., Sridhar, M., Suresh, K., Banat, I.M., Venkateswar Rao, L., 2000. Bioresour. Technol. 72 (1), 43–46.

Lichstein, H.C., Soule, M.H., 1944. J. Bacteriol. 47 (3), 221–230.

McCann, J., Choi, E., Yamasaki, E., Ames, B.N., 1975. Proc. Natl. Acad. Sci. U.S.A. 72 (12), 5135–5139.

Morris, K., Zhao, H., John, R., 2005. Aust. J. Chem. 58 (4), 237–245.

Neufeld, T., Biran, D., Popovtzer, R., Erez, T., Ron, E.Z., Rishpon, J., 2006. Anal. Chem. 78 (14), 4952–4956.

Oliver, D.B., Cabelli, R.J., Dolan, K.M., Jarosik, G.P., 1990. Proc. Natl. Acad. Sci. U.S.A. 87 (21), 8227–8231.

Pemberton, R., Rawson, F., Xu, J., Pittson, R., Drago, G., Griffiths, J., Jackson, S., Hart, J., 2010. Microchim. Acta 170, 321–330.

Peng, L., You, S.-J., Wang, J.-Y., 2010. Biosens. Bioelectron. 25 (5), 1248–1251.

Rawson, D.M., Willmer, A.J., Turner, A.P.P., 1989. Biosensors 4 (5), 299–311.

Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T., Lovley, D.R., 2005. Nature 435 (7045), 1098–1101.

Richter, H., McCarthy, K., Nevin, K.P., Johnson, J.P., Rotello, V.M., Lovley, D.R., 2008. Langmuir 24 (8), 4376–4379.

Rogers, K.R., 2006. Anal. Chim. Acta 568 (1–2), 222–231.

Schaetzle, O., Barriere, F., Baronian, K., 2008. Energ. Environ. Sci. 1 (6), 607–620.

Sikkema, J., de Bont, J., Poolman, B., 1995. Microbiol. Rev. 59 (2), 201–222.

Tender, L.M., Reimers, C.E., Stecher III, H.A., Holmes, D.E., Bond, D.R., Lowy, D.A., Pilobello, K., Fertig, S.J., Lovley, D.R., 2002. Nat. Biotechnol. 20 (8), 821–825.

Timur, S., Anik, U., Odaci, D., Gorton, L., 2007. Electrochem. Commun. 9 (7), 1810–1815.

Vostiar, I., Ferapontova, E.E., Gorton, L., 2004. Electrochem. Commun. 6 (7), 621–626.

Walker, A.L., Walker, J.C.W., 2006. J. Power Sources 160 (1), 123–129.

Zakeeruddin, S.M., Fraser, D.M., Nazeeruddin, M.K., Grätzel, M., 1992. J. Electroanal. Chem. 337 (1–2), 253–283.