This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
We report the first example of a biofuel cell operating in organic solvents. The cell utilises glucose oxidase and bilirubin oxidase immobilised on nanoporous gold. The power output of the cell decreases with increasing solvent hydrophobicity in the alcohols, methanol (MeOH), ethanol (EtOH), 1-propanol (PrOH), 1-butanol (BuOH) and 1-pentanol (PeOH).

Enzymatic biofuel cells (BFCs) that utilise oxidoreductases to generate electrical energy are of interest due to their potential applications as autonomous power supplies\(^1,2\). A wide range of reports have described the development of miniaturized BFCs with extended lifetimes and increased power densities, focussing on screening enzymes from a variety of sources, developing more efficient methods of immobilization, the use of a range of electrode materials and the deployment of enzyme cascades\(^3,4\). Due to potential applications in biomedical devices, the properties of BFCs are invariably examined in physiological conditions, with no reports on their use in nonaqueous solvents.

Enzymes can preserve their native structures and retain catalytic activity in nonaqueous solutions\(^5\). While the catalytic activity of enzymes in such media can be significantly lower than in aqueous solution\(^6\), the use of enzymes in such media has a number of advantages that include increased substrate solubility, increased thermal stability, suppression of side reactions that can occur in water and changes in enzymatic selectivity\(^7,8\). Enzymes are insoluble in nonaqueous media and as a consequence, electrochemical studies of enzymes in such media require that the enzyme be immobilised on the electrode. Studies on enzyme modified electrodes in organic solvents have focussed on their use in biosensors and in probing the kinetics of catalytic activity in nonaqueous solutions and thermodynamics of the redox process. Several reports that enzymes in organic solvents have higher selectivity for substrates and products\(^9,10,11\), properties such as viscosity\(^12\) and the dielectric constant\(^13,14\) can affect the enzymatic activity and specificity\(^15\)

Herein, we describe the properties of a well-studied BFC based on glucose and O\(_2\) using glucose oxidase (GOx, Aspergillus niger) and bilirubin oxidase (BOD, Myrothecium verrucaria) entrapped with the osmium polymers \([\text{Os}(4,4′-\text{dimethyl-2,2′-bipyridine})_2\text{(polyvinylimidazole)}\text{Cl}]+/2^+\) (Os(dmbpy)_2PVI) and \([\text{Os}(2,2′-\text{bipyridine})_2\text{(polyvinylimidazole)}\text{Cl}]+/2^+\) (Os(bpy)_2PVI) (Fig. 1A)\(^16\). Poly(ethylene glycol)diglycidyl ether (PEGDGE) was used as the cross-linking agent. The enzyme/redox polymer mixtures were immobilised onto dealloyed nanoporous gold (NPG) electrodes (details in ESI\(^\dagger\))

The catalytic activities of the NPG/Os(dmbpy)_2PVI/GOx and NPG/Os(bpy)_2PVI/BOD electrodes were separately studied in a three-electrode cell. Cyclic voltammograms (CVs) of NPG/Os(dmbpy)_2PVI/GOx in 0.1 M pH 7.0 phosphate buffer (PBS) exhibited a pair of well-defined redox peaks corresponding to the conversion of Os\(^{3+}/3^+\) at a low scan rate of 5 mV s\(^{-1}\) (Fig. 1B, solid line). The peak potential separation, \(\Delta Ep\), of 15 mV, was indicative of a rapid and reversible electron transfer process. Upon addition of 5 mM glucose, a sigmoidal-shaped curve, characteristic of the biocatalysis of oxidation of glucose, with an onset potential of -0.1 V, was obtained (Fig. 1B, dashed line). The response current density, \(j_{\text{response}}\), defined as the difference between the catalytic and background current density was 54 \(\mu\text{A cm}^{-2}\) in PBS. The electrode was then transferred into acetonitrile (ACN) containing 5% added buffer. A reversible redox curve (Fig. 1C, solid line) with a \(j_{\text{response}}\) of 2.2 \(\mu\text{A cm}^{-2}\) (4% of the original activity in PBS, Fig. 1C, dashed line), with an increased onset potential of -0.03 V, was obtained. The catalytic response in both PBS and ACN was further confirmed by chronoamperometry, with a catalytic current clearly evident (Fig. S1). This agrees with previous reports that enzymes in organic solvents...
media possess only a fraction of the catalytic activity observed in water\(^1\) with the decreased activity arising from a range of effects\(^2\) (e.g. decrease in molecular flexibility\(^2\)), reduction in the amount of water bound to the enzyme). There was no perceptible change in the cathodic peak potential. It is noteworthy that the response of the electrode was largely the amount of water bound to the enzyme). There was no retained on reimmersion in aqueous buffer solution (data not shown). The operational stability of BFC in PBS indicated that GOx had not been denatured in the organic solution.

NPG/Os(bpy)\(_2\)PVI/BOD cathodes also showed a pair of reversible and well-defined redox peaks in \(N_2\) bubbled PBS (Fig. 1D, solid line). An initial \(j_{\text{response}}\) of 123 µA cm\(^{-2}\) and an onset potential of ~0.43 V in \(O_2\) bubbled aqueous solution were obtained (Fig. 1D, dashed line). On switching to 95% ACN, a significantly lower \(j_{\text{response}}\) of 5 µA cm\(^{-2}\) (Fig. 1E, dashed line) was observed, while the onset potential of \(O_2\) reduction decreased to approximate 0.33 V. Chronoamperometric data confirmed the activity of the enzyme in ACN (Fig. S2). The recovery of activity (\(j_{\text{response}}\) of 105 µA cm\(^{-2}\), 85% of initial response) in PBS indicated that BOD had not been significantly denatured on exposure to 95% ACN (Fig. 1D, dotted line). No catalytic response was observed in the absence of the enzyme (Fig. S3).

Based on the above results, GOx and BOD modified anodes and cathodes were subsequently assembled into BFCs (Fig. 1A), and the response of the cell monitored by linear sweep voltammetry (scan rate of 1 mV s\(^{-1}\)). The BFC displayed an open circuit voltage (OCV) of 0.56 V (the difference of the onset potentials associated oxidation of glucose and reduction of \(O_2\)), a maximum current density of 21.2 µA cm\(^{-2}\), and a maximum power density of 3.65 µW cm\(^{-2}\) at a potential of 0.21 V in \(O_2\) bubbled PBS containing 5 mM glucose (Fig. 1F, solid line). On storage at 4°C, the cell retained 75% of the initial response (Fig. S5). A minor decrease of 15% in the OCV was observed after storage for 60 h.

On replacement with 95% ACN, the performance of the BFC decreased, with an OCV of 0.36 V, a maximum current density of 7.11 µA cm\(^{-2}\), and a maximum power density of 0.47 µW cm\(^{-2}\) at 0.12 V (Fig. 1F, dashed line). The decrease in power arises both from changes in OCV and in current density (enzyme activity). The response of the cell was retained on reimmersion in PBS, (OCV of 0.56 V), and a maximum power density of 3.44 µW cm\(^{-2}\) (94% of the original response, Fig. 1F, dotted line). Leakage of redox polymer from the electrode surface was mainly responsible for the loss response\(^17\) as evidenced by the decrease in the peak current in a blank buffer solution (data not shown). The operational stability of BFC in 95% ACN was examined, with a half-life of ca. 3 h (Fig. S4).

The response of the BFCs was examined in solutions with varying water content (1-5% (v/v)) in ACN (Fig. 2A). The maximum power density 0.47 µW cm\(^{-2}\) was obtained with 5% added buffer decreasing to 0.13µW cm\(^{-2}\) in 99% ACN, indicating as expected, that the enzymes are more active at higher water content. The response of the BFC was examined in a series of solvents with 5% v/v added buffer (Fig. 2B). The maximum power density was obtained in methanol and decreased in the sequence methanol < ethanol< propanol< butanol < pentanol. The response in these solvents decreased with increasing solvent hydrophobicity in an approximately linear manner (Fig. 2C). The response in ACN and acetone (AC) was lower than in the alcohols and did not follow this linear relationship. This may ascribed to the complexity of the integration of two different enzymes immobilized in the polymer matrix. Generally, the BFC had a higher power density in organic solvents with lower values of log \(P\). Similar trends were reported for single enzyme electrodes\(^14, 23\). The trend observed here is likely to arise from solvent based interactions at the enzymes active sites with either the enzymatic substrates or with the redox polymers.
In conclusion, we describe the assembly of a membraneless BFC that coupled an NPG/Os(bpy)$_2$PVI/BOD biocathode that operates in organic solvents. More importantly, both bioelectrodes displayed reversible recovery of their initial activities in PBS after operation in organic solutions. A well-defined trend with the maximum power density decreasing with increasing log $P$ was obtained in straight-chain monohydric alcohols. The use of this BFC is limited to a small range of solvents due to the low solubility of glucose in nonaqueous media (generally not higher than 5 mM glucose in 95% organic solvent). Applications for the BFC described here may be possible in nonaqueous solvents with low water content. Future work will focus on the use of oxidases that utilise substrates (e.g. EtOH) with high solubility in such media.

This work was financially supported by the European Commission (FP7-PEOPLE-2013-ITN 607793 “Bioenergy”). X. Xiao acknowledges a Government of Ireland Postgraduate Scholarship. We thank Prof. Donal Leech for the gift of redox polymers.

Notes and references