Continuous power generation from glucose with two different miniature flow-through enzymatic biofuel cells

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A R T I C L E   I N F O

Article history:
Received 5 January 2015
Received in revised form 17 February 2015
Accepted 24 February 2015
Available online 26 February 2015

Keywords:
Enzymatic biofuel cells
Bioenergy
Highly porous gold
Flow-through
Glucose oxidase
Laccase

A B S T R A C T

Enzymatic biofuel cells (EBFCs) can generate energy from metabolites present in physiological fluids. They represent an attractive alternative to lithium batteries to power implantable devices, as they work at body temperature, are light and easy-to-miniaturise. To be implantable in blood vessels, EBFCs should not only be made of non-toxic and biocompatible compounds but should also be able to operate in continuous flow-through mode. The EBFC devices reported so far, however, implement carbon-based materials of questionable toxicity and stability, such as carbon nanotubes, and rely on the use of external redox mediators for the electrical connection between the enzyme and the electrode. With this study, we demonstrate for the first time continuous power generation by flow through miniature enzymatic biofuel cells fed with an aerated solution of glucose and no redox mediators. Non-toxic highly porous gold was used as the electrode material and the immobilisation of the enzymes onto the electrodes surface was performed via cost-effective and easy-to-reproduce methodologies. The results presented here are a significant step towards the development of revolutionary implantable medical devices that extract the power they require from metabolites in the body.

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1. Introduction

Millions of patients worldwide suffer from serious diseases such as bradycardia, fibrillation or diabetes, and consequently require active medical implants (WHO 2014). Size, weight, and reliability are fundamental characteristics of these devices, and are typically determined by the power source utilised. Active medical implants are traditionally powered by lithium batteries, which are heavy and difficult to miniaturise, leading to disproportionately large energy sources compared to the systems that they power (Bazaka and Jacob 2012). The search for alternative power sources, which are light, non-toxic, and easy-to-miniaturise, is therefore crucial.

Enzymatic biofuel cells (EBFCs) are power sources that can be employed in the human body (Barton et al. 2004). EBFCs are a specific type of fuel cell that implements redox enzymes as catalysts at the anode and cathode. They can mimic many of the metabolic pathways found within living cells (in particular the oxidation of glucose), and thus produce power from energy sources naturally found in biological fluids at body temperature (Cosnier et al. 2014). EBFCs have also the added benefit of producing the same waste products as the living organism that hosts them, and could thus use established waste metabolism routes to dispose of the by-products produced during the production of power. These features make EBFCs an attractive alternative to lithium batteries.

For the purpose of in vivo use, EBFCs could either be implanted in soft tissue or in blood vessels (Barton et al., 2004; Cinquin et al., 2010; Kerzenmacher et al., 2008). In the case of devices to be implanted in the soft tissue, the system is limited by lower fuel and oxidant concentrations (glucose and oxygen), and is thus reliant solely on diffusion for the supply and removal of reactants and waste products respectively. Devices designed for use in blood vessels, would instead exhibit continuous flow through operation. In this case, the EBFC would benefit by having higher concentrations of glucose and oxygen, continuously supplied by the flow of blood. The continuous flow could however lead to enzyme leaching from the electrodes, and could interfere with the electron transfer between enzymes and the electrode surface (Kerzenmacher et al., 2008).

Currently the practical application of EBFCs is prevented by several major hindrances, the most significant of which are poor stability and extremely low power yields (Wei and Liu, 2008). However, the poor stability is not necessarily caused by the deactivation of the enzymes used, but rather by a decay in the efficiency of electron transfer between the enzymes and the electrode surface. This is due to the fact that most EBFCs reported so far rely on mediated electron transfer (MET) techniques, which typically require foreign redox active particles to transfer electrons between
the active site of the enzyme and the electrode surface (Cooney et al., 2008; Degani and Heller, 1987; Minteer et al., 2007). Over time, these free moving redox mediators can leach out from the fuel cell’s electrodes causing a decay in power output.

The development of new enzyme immobilisation techniques in recent years, which achieve direct electron transfer (DET) between the enzyme and the electrode, together with major advances in material sciences allowing for much higher enzyme loading, and advances in electronics leading to ultra-low power medical devices (with a typical pacemaker now only requiring 10 µW) have opened new perspectives and reinforced the interest for EBFCs (Cosnier et al., 2014).

Power generation by EBFCs from biological fluids has been recently reported (Kim et al., 2006; Rincón et al., 2011; Sokic-Lazic and Minteer, 2009; Togo et al., 2007). Nonetheless, there is currently very little reported on fully-fledged devices capable of continuous operation under physiological conditions. The majority of devices reported so far show the use of separate enzymatic electrodes simply placed in the living organism and externally wired. In the case of continuous flow-through operation, these devices still rely on the use of mediators in the feed solution. There are no reported EBFCs that exhibit continuous flow-through operation without mediators for the potential use in line with blood vessels. Instead the few studies that have progressed to the development of devices capable of sustained operation have thus far focused on implantation in the soft tissue of animals (Castorena-Gonzalez et al., 2013; Cinquin et al., 2010; Halámková et al., 2012; MacVittie et al., 2013; Rasmussen et al., 2012; Sales et al., 2013; Szczupak et al., 2012).

All of these reported studies rely on the use of carbon-based electrodes. In particular, carbon nanotube (CNT) aggregates have been widely implemented, since they allow DET with glucose oxidase (GOx), which is the most prevalent enzyme used for glucose oxidation at the anode of EBFCs (Anthony et al., 2002; Ivnitski et al., 2006; Liu et al., 2005). However, the long term toxicity and stability of CNTs is still unknown. CNTs have been reported to cause the destruction of T lymphocyte cells in mammals (Bottini et al., 2006). Direct contact between the CNTs electrode and physiological fluids should therefore be carefully prevented in implantable applications. Some researchers have tackled this obstacle by enclosing their devices in semi-permeable membranes which would prevent direct contact between the electrodes and white blood cells (Cinquin et al., 2010). This may, however, not be possible when designing a device for implantation in or around blood vessels, since the device would have to be small enough to cause minimal disruption to normal blood flow. The search for electrode materials, as an alternative to carbon-based systems is therefore critical for implantable EBFCs. In this context, highly porous gold (hPG) electrodes are a promising alternative. These non-toxic electrodes have remarkable properties, such as high conductivity, large surface area, three-dimensional open porosity, and biocompatibility. Moreover, their large surface area and foam-like morphology make hPG electrodes the ideal support for enzyme immobilisation at high loadings as previously demonstrated by this group and others (Chen et al., 2012; du Toit and Di Lorenzo, 2014b; Hakamada et al., 2012; Xiao et al., 2013).

In this study, we demonstrate for the first time sustained power production from continuous flow-through enzymatic biofuel cells (CFEBFCs) for periods of up to one month without the use of external redox mediators. We also demonstrate a simple and low cost methodology for fast prototyping of CFEBFCs using 3D printed moulds, as well as the electrochemical production of hPG electrodes without the need for potentiostatic control. Moreover, GOx, implemented at the anode, is electrostatically immobilised onto the hPG surface via the simple and easy-to-reproduce method that we have previously reported (du Toit and Di Lorenzo, 2014b).

2. Experimental

2.1. Materials

GOx from Aspergillus niger, laccase (LAC) from Rhus vernicifera, and all other reagents used were of analytical grade and purchased from Sigma-Aldrich. Unless stated otherwise, all the aqueous solutions used were prepared with reverse osmosis purified water. Saturated calomel electrodes (SCE) were purchased from ijCambridge Ltd. Platinum wire was purchased from Cookson Precious Metals Ltd. Polydimethylsiloxane (PDMS, Dow Corning Sylgard 184) was purchased from Ellsworth Adhesives.

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 Na2HPO4, 2 mM KH2PO4. The pH of this solution was then adjusted to 7 with the drop wise addition of 1 M solutions of HCl and NaOH.

All potentiostatically-controlled electrochemical processes were conducted using the Autolab PGSTAT128 N (Metrohm, UK) potentiostat. The load on the fuel cells was controlled using a Cropico variable resistance box (RS Components) and the potential difference was monitored and recorded using a Picolog ADC-24 multichannel data logger. The moulds for the PDMS replicas were 3D printed in polyactic acid using a Makerbot Replicator.

2.2. Deposition of hPG onto platinum wires

The hPG was fabricated with a two-stage process similar to the process previously described (du Toit and Di Lorenzo, 2014a). Briefly, controlled lengths of platinum wire electrodes were immersed in an electrolyte consisting of 0.1 M HAuCl4 and 1 M NH4Cl. Gold was deposited in two steps. For platinum wire lengths of 1 cm or less, the working potential was first gradually stepped down to −4.0 V (vs. SCE) over a period 10 s using the Autolab PGSTAT128N (Metrohm, UK) potentiostat. This potential was then maintained for a further 10 s. For platinum wire lengths greater than 1 cm (where the required deposition current exceeded the capabilities of the potentiostat), a simple two-electrode system was used with a second platinum wire as the counter electrode. This time the potential applied across the two electrodes was gradually stepped down to −10 V and then maintained at this potential for 10 s using the Basetech BT-305 variable bench-top power supply unit. This was done in accordance with the actual potential applied across the working electrode and counter electrode when using a potentiostat with a three-electrode setup.

The morphology of the resulting electrodes was characterised using a Hitachi S-4300 field emission scanning electron microscope (FESEM).

2.3. Enzyme immobilisation onto hPG electrodes

GOx and LAC were immobilised on the hPG anode and cathode respectively to facilitate the oxidation of glucose at the anode and the reduction of oxygen at the cathode according to the following reactions:

\[ \text{C}_6\text{H}_{12}\text{O}_6 \xrightarrow{\text{GOx}} \text{C}_6\text{H}_5\text{O}_6 + 2e^- + 2H^+ \]

\[ \frac{1}{2} \text{O}_2 + 2e^- + 2H^+ \xrightarrow{\text{LAC}} \text{H}_2\text{O} \]

GOx was electrochemically adsorbed onto the prepared hPG wire electrodes using a process based on the method described previously (du Toit and Di Lorenzo, 2014b). Simply, 6 CV scans were conducted between 0.42 V and 0.60 V (vs. SCE) at a scan rate...
of 1 mV s\(^{-1}\), in a PBS solution containing 15 mg ml\(^{-1}\) GOx (approximately 270 U ml\(^{-1}\) as per activity rating of manufacturer). LAC was immobilised using a multistage method based on a method previously reported by this research group and a thiol blotting method reported by other researchers for the immobilisation of laccase from \textit{Trametes versicolor} (du Toit and Di Lorenzo, 2014b; Pita et al., 2011). The surface of the hPG wire 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 electrolyte containing 10%\(v/v\) EtOH and 0.1 M KCl. Afterwards, the electrodes were transferred to a solution containing 10 mM 6-mercapto-1-hexanol and left overnight at room temperature. The following morning 1 mg of LAC (270 U as per activity rating of manufacturer) was added to 0.38 ml of a 50 mM sodium periodate solution. After 30 minutes of incubation at room temperature this LAC solution was made up to 1.0 ml by the addition of 0.62 ml of a 100 mM Na\(_2\)HPO\(_4\) solution. The amino-phenyl and thiol functionalised hPG electrodes were then submerged in this LAC solution for 90 min. Finally the electrodes were taken from the LAC solution and, after repeated rinsing in a 10 mM 2-morpholinoethanesulfonate (MES) buffer, were submerged in a solution containing 10 mM MES, 20 mM N-hydroxysuccinimide and 40 mM 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide for 2 h before being rinsed with and stored in PBS until required.

### 3. Results and discussion

#### 3.1. Enzymatic biofuel cell fabrication

Two different CFEBFC designs were considered in this study. In the first design, the anode and the cathode (1 cm long each) were fit in two parallel channels, each with a 3 mm × 3 mm cross-sectional area, separated by a PDMS wall (Fig. 1A). The second design was characterised by a single channel (10 mm × 3 mm cross-sectional area) containing the two electrodes, which were positioned so that the electrolyte would flow over the cathode and the anode sequentially (Fig. 1B). In this design, the electrode’s projected surface area was increased by repeatedly folding 3 cm long electrodes to occupy a total cross-sectional area of 1 cm by 0.5 cm. The negative of the CFEBFC was 3D printed and then used as a mould over which PDMS was cast to create the final fuel cell design (Fig. 1C). Once the enzymatic electrodes were inserted, the PDMS structure was clamped between two pieces of acrylic plastic (Fig. 1D).

The electrodes were manufactured by directly electrodedepositing hPG films onto platinum wires via a hydrogen bubble template (du Toit and Di Lorenzo, 2014a). This technique relies on the simultaneous deposition of gold and evolution of hydrogen gas, and thus is reliant on relatively high deposition potentials and currents. The deposition is therefore easy to achieve and control with a potentiostat in the case of small test electrodes (such as for the electrodes used in the parallel channels design). When larger surfaces are used, such as the platinum wires used in the single channel design, potentiostatic control is, however, harder to maintain. The use of a DC power supply as an alternative to the potentiostat was therefore investigated to simplify the established hPG deposition method. Fig. 2 compares the FESEM images of the
electrodes used in the two different CFEBFC configurations obtained either via a potentiostatically controlled electrodeposition (Fig. 2A), or by using a DC power supply (Fig. 2B). As shown, the two methodologies led to the same porous structure. This is a significant move towards the low cost production of hPG electrodes on a larger scale.

3.2. Performance of the CFEBFCs

The CFEBFCs were tested by continuously feeding them with an aerated PBS solution containing 27 mM glucose, at a rate of 0.35 ml min$^{-1}$ and under the constant temperature of 37°C as shown in Supplementary data.

The performances of the two CFEBFCs were firstly compared on the basis of their polarisation and power curves. These were obtained by step-increasing the resistance applied across the fuel cell between 1 kΩ and 1 MΩ, as well as by measuring the open circuit potential (OCP). Fig. 3 shows the results obtained. A marked difference in performance (either specific by electrode surface area, or overall) was observed between the two CFEBFCs. The power and current density obtained with the single channel CFEBFC (Fig. 3B) are in fact much lower than the case of two channels CFEBFC (Fig. 3A).

Moreover, in the case of the single chamber CFEBFC, a drop in OCP of approximately 90 mV was observed. The potential interference of reaction intermediates, such as H$_2$O$_2$, could be the cause of the poorer performance of the single chamber CFEBFC. H$_2$O$_2$ is produced when reduced GOx comes in contact with oxygen as described by the following reactions:

$$\text{CH}_6\text{H}_12\text{O}_6 + \text{GOx} \rightarrow \text{CH}_6\text{H}_10\text{O}_6 + \text{GOx}, \text{H}_2$$

$$\text{GOx}, \text{H}_2 + \text{O}_2 \rightarrow \text{GOx} + \text{H}_2\text{O}_2$$

This poses no problem at the GOx electrode as H$_2$O$_2$ is can be involved in improving electron mediation between the enzyme’s active site and the hPG surface, according to the following reaction:

$$\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}^+ + 2\text{e}^-$$

The back diffusion of H$_2$O$_2$ can however cause interference at the LAC electrode by causing an electrochemical short-circuit. Laccase could also be deactivated by H$_2$O$_2$ as previously reported (Milton et al. 2013). Enzyme migration from one electrode to another could also be a risk. If, as a consequence of the continuous
flow, the enzyme from the first electrode is released in the solution it could be absorbed into the porous structure of the following electrode with the result of short-circuiting the electricity production. In our case, however, this risk is believed to be minimal since the electrodes were rinsed thoroughly prior to use to remove any poorly attached enzyme. Moreover, the single channel CFEBFC was set-up so that the feed would first pass over the cathode and then to the anode. This was done on the assumption that, the LAC, covalently attached to the electrode contrarily to GOx, presented minimum risk of leaching.

The specific power output by the geometric surface area of an electrode is the most common metric used for determining fuel cell performance, however it does not always have practical relevance. In this study, the highest specific power output by anode surface was obtained with the parallel channels CFEBFC; with a value (1.6 μW cm⁻²) more than two times greater than the single channel CFEBFC (0.7 μW cm⁻²). Nonetheless, since the parallel channels CFEBFC is reliant on the use of a physical divider between electrodes, it has a larger overall volume relative to the electrode size. A more practical evaluation of the CFEBFCs’ performance should therefore consider not only the surface area of the electrodes but also the overall size and shape of the constructed fuel cells.

The overall polarisation of a fuel cell can also give a misleading characterisation of the fuel cells since it only takes a snapshot of their performance. The power produced by the two types of fuel cells was therefore investigated during continuous operation.

Fig. 4 shows the average continuous power production from the two CFEBFC designs during the first 24 h of operation. In each case, the fuel cell load was chosen so as to maximise power output (30 kΩ and 10 kΩ for the parallel channel and the single channel CFEBFC respectively). According to the parameter used for comparison, the two designs give apparently controversial results. When the specific power output is considered, the parallel channel CFEBFC consistently outperforms the single channel CFEBFC (Fig. 4A). On the contrary, when the total power produced is taken into account, the single channel system presents better performance than the parallel channel system (Fig. 4B). This suggests that there is a potential trade-off between the CFEBFCs specific power output by area and its specific power output by volume.

Alternatively, assuming that H₂O₂ is the cause for the lower power density by electrode area for the single chamber system, the use of a physical divider could be overcome by operating the cell at flow velocities high enough to counteract H₂O₂ diffusion. The CFEBFCs were in fact fed with the same volumetric flow rate (0.35 ml min⁻¹). Due to their different cross-sectional areas, the flow velocities in the CFEBFCs were however different: 1.94 cm min⁻¹ and 1.17 cm min⁻¹ for parallel channels CFEBFC and single channel CFEBFC respectively. This difference could affect the mass transfer both from the bulk of the solution to the enzymes on the electrodes, and subsequently between the enzymes and the hPG. The impact that different flow velocities have on the power output generated by the two CFEBFCs was consequently investigated.

Fig. 5 shows the power output produced by the two CFEBFCs at increasing velocities of flow. As shown, differences in flow velocity had little effect on the parallel channels CFEBFC. On the other hand, exponential increases of the flow velocities had a significant impact on the performance of the single chamber CFEBFC. It has been reported that the GOx’s apparent $K_m$ (which inversely indicates enzymes’ affinity for glucose) increases with flow rate.
A general decay in power output is therefore expected at high flow rates. This decay was observed in the case of the parallel channel CFEBC. Contrarily, in the case of the single channel CFEBC the power output increased at flow velocities higher than 0.04 cm min$^{-1}$. This result supports the hypothesis that H$_2$O$_2$ back diffusion is one of the causes of poor performance at low flow rates. The high flow rates could also provide a better supply of reactants to hard-to-reach regions of the pleated electrode surfaces owing to increased turbulent mixing. Higher flow velocities are however associated with a much higher throughput of fuel, with exponential increases in flow rate yielding only minor increases in performance. An increase in the flow velocity of one order of magnitude (from 0.03 cm min$^{-1}$ to 0.7 cm min$^{-1}$) led in fact to a power output only 0.75 times higher. Future work is, however required to take a more in-depth look at the flow phenomena in the two cells and further investigate and quantify the effects of flow rate on their operation.

Although the initial power output generated by enzymatic biofuel cells is frequently reported to be sufficient for ultra-low power implantable medical devices, their long-term stability during continuous operation is very poor. Many enzymatic fuel cells reported so far exhibit in fact a rapid decay in performance, with most lasting only a few hours (Castorena-Gonzalez et al., 2013; Cinquin et al., 2010; MacVittie et al., 2013; Togo et al., 2007). In order to determine the long-term stability of the CFEBC it was decided to record the power output during continuous operation for one month. To the best of our knowledge, this is the first study performed over such a period of time with an enzyme immobilised EBFC operating in continuous flow with no addition of external mediators.

The parallel channel fuel cell was selected for this analysis since it produced a more stable power output at low flow rates with low risk for H$_2$O$_2$ diffusion from the anode to the cathode. Fig. 6 shows the continuous power production from the parallel channel EBFC over a period of 30 days. As shown, during the first 48 h of operation, the CFEBC exhibited a growth in the power produced, before entering a general decay phase. The fluctuations in the power output observed are attributed to the evolution of gas bubbles within the fuel cell. Although every effort was taken to prevent this happening by using drips prior to feeding the CFEBC (see Supplementary data), some gas bubbles would still evolve during long-term continuous operation. A sharp drop in power was observed after 20 days of continuous operation, which continued until the 23rd day when the CFEBC was washed with a fresh PBS solution. This suggests that there might have been some contamination in the feed that inhibited the power production.

Regardless of the short-term fluctuations in the power output from the CFEBC, a strong trend was maintained with an apparent constant rate of decay. From this decay, the half-life of the fuel cell was determined to be approximately eight days. Since there is no other research reported to have used a continuous flow through enzymatic biofuel cell or to have even tested the long-term stability for over 30 days, it is difficult to make comparisons to the existing state-of-the-art on the subject. However, it can be reported that this CFEBC has a half-life which is in line with the half-life of free laccase in solution (3–8 days under optimal conditions (Zille et al., 2003)), and also with that of a carbon-based GOx-LAC fuel cell (approximately six days based on continuous operation for 72 h (Chen et al., 2001)). Since GOx has reported half-lives in excess of 200 days, the LAC electrode is likely to be the limiting electrode and the cause of the decay in power output (Rando et al., 1997). This result is very promising since the majority of enzyme-immobilised devices exhibit much faster decays in power output owing to deactivation or detachment of enzymes. The fact that the decay in power output is in line with the decay of the enzymes themselves suggests that the methods employed for immobilisation are stable over long periods of time. The long-term stability of this CFEBC could therefore be improved by simply increasing the stability of the enzymes used, either through protein engineering, or through cross-linking with stabilising polymers.

### 4. Conclusions

In this study we report continuous power generation from two innovative continuous flow enzymatic biofuel cell configurations that implement highly porous gold electrodes and glucose oxidase and laccase as the catalysts. The fabrication methodology here presented is cost-effective, easy-to-reproduce and fast, allowing for large-scale production and for further in-depth investigation on optimal electrode configuration and flow channel design.

By comparing the performance of the two CFEBCs, we demonstrated that the power output generated is influenced by the channel designs; shape and size of the electrodes; and flow rate through the fuel cells. The design and operation of the CFEBC can therefore be customised depending on the intended use. If miniaturisation is a major requirement, then using larger electrodes in a single channel device will maximise power output, but this will require high flow rates. Conversely, if space is not limited, then the specific power output can be maximised by physically separating the anode and cathode and thus increasing the relative size of the fuel cell.

This study also reports for the first time the long-term stability of a CFEBC under continuous operation. Typically enzymatic biofuel cells exhibit lifetimes of only a few hours owing to the detachment or deactivation of enzymes, or commonly due to the leaching of redox mediators. In this study, however, the power output produced by the CFEBC is stable during the first 24 h of operation. Furthermore, though decay in performance is observed during continual use over many days, the half-life of decay is in line with the state-of-the-art decay of the enzymes implemented under extremely favourable conditions (e.g. aqueous conditions).

### Acknowledgments

The authors acknowledge: Prof Frank Marken, University of Bath, for valuable discussions and EPSRC (EP/K031597/1) for funding.
Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.02.036.

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