A BIOLOGICALLY-INSPIRED ELECTRO-CHEMICAL REFERENCE ELECTRODE

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ABSTRACT

The paper report a unique biologically inspired electro-chemical reference electrode based on regulating the breathing of bacteria. Some species of bacteria, named exoelectrogen, have the capability of extracellular electron transfer, which is the transfer of electrons to a solid electron acceptor outside their membrane. We find that it sets the solid electron acceptor at a stable electrochemical potential, which can be used as reference electrode. We pattern thin film platinum as electron acceptor and grow exoelectrogenic biofilm on it. By performing colorimetric analysis of the individual ions in the anolyte solution, we confirmed that the potential of the reference electrode, ~ -0.5 V versus the Ag/AgCl in 3M NaCl, arises from the electrochemical potential of the reaction. The biologically-inspired reference electrode demonstrates a stability of ±4.2 mV/day for two days. It is integrated in a MEMS microbial fuel cell (MFC) to characterize its electrochemical characteristics.

INTRODUCTION

Nowadays biological-inspired devices and systems have become an active area of research. Many biological-inspired devices have been reported in the past decade, including biologically-inspired digital cameras[1], biologically-inspired artificial lungs[2], biologically-inspired camouflage and display for soft machines[3] and biologically-inspired supercapacitors[4]. These biologically inspired devices and systems demonstrated not only technological novelty but also good performance enhancement.

In the past 50 years, microsystems and lab-on-a-chip have been on active research through successful miniaturization of sensors, actuators, and systems[5, 6]. Reference electrodes, which maintain a stable potential, have been widely implemented in electrochemistry, such as fuel cells and electrochemical sensors[7, 8], has also been sought for miniaturization as conventional references are too bulky to be implemented in microsystem and lab-on-a-chip setups. However, implementing a microfabricated reference electrode in microsystems / lab-on-a-chip has been a long sought challenge to date, primarily due to the poor long-term stability. For instance, Polk et al. microfabricated silver/silver chloride thin films to be a reference electrode, yet it marked rather poor stability of ±2.88 mV/day for only 1.4 hours [9]. Another attempt deposited silver chloride nanosheets on silver wire, yet, again the stability of ±3.2 mV/day for 15 hours needs substantial improvement [10]. Because reference electrodes need to provide a stable electrochemical potential for extended period of time, when being implemented in electrochemical applications such as microbial electrochemical techniques, environmental monitoring, *etc.*, the microfabricated references electrode to date falls short. As a substitution, currently bulky conventional reference electrode is implemented in our MEMS microbial electrochemical research, which greatly increases size of the overall device. It directly motivates our work: to study a microfabricated reference electrode with a long-term stability.

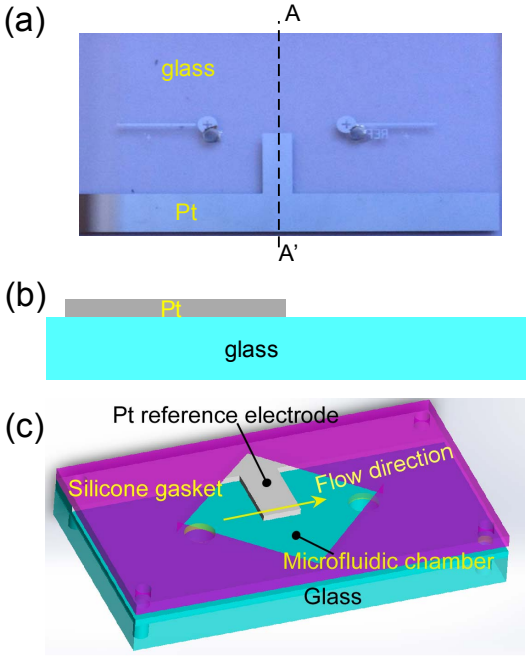

Figure 1. (a) Optical image of the Pt reference electrode on a glass slide fabricated by one step lift off; (b) Cross-sectional view of (a) across A-A'; (c) Schematic of implementing the reference electrode in a microfluidic chamber.

FABRICATION AND TEST SETUP

Reference Electrode Fabrication

The biological-inspired reference electrode was fabricated by patterning a thin film Platinum (100nm) by lift-off to form an electrode. First, six through holes were drilled on a rectangular glass substrate (46 mm × 26 mm × 1 mm), one as inlet, one as outlet for the reference electrode and the rest four as screw holes for fixing. Then the glass substrates were cleaned by piranha solution cleaning. Subsequently a layer of 4 μm thick photoresist was patterned on the glass slide, and a layer of Pt (100 nm) was deposited using an electron beam evaporator. The reference electrode was patterned by lifting Pt off in acetone. The optical microscopy and cross-sectional images of the reference electrode is shown in Figure 1 (a, b).

The cathodes in this paper were made of gold. A layer of Au (200 nm) deposited using a magnetron sputter on a glass substrate (46 mm × 26 mm × 1 mm), which is used as a cathode.

Device Assembly

The reference electrode, cathode, proton exchange membrane, 250 µm thick silicone gaskets, nanoports, bolts and nuts were assembled to build the device for growing *Geobacter sulfurreducens* on the reference electrode and for characterizing the electrochemical. First, nanoports were glued to the inlet and outlet of reference electrode and cathode glass substrates. Second, two silicone gaskets were patterned with a hole of 1 cm × 1 cm at the center, in order to define the anode and cathode chamber volume of both 25 µL. Third, a rectangular PEM was cut, and reference electrode, cathode, PEM and silicone gaskets were assembled.

***Geobacter Sulfurreducens* Enriched Biofilm Formation**

Geobacter sulfurreducens enriched bacterial community originally from anaerobic-digestion sludge was used as inoculum for the biologically-inspired reference electrode. The inoculum was obtained from an acetate-fed microbial electrolysis cell (MEC). The anolyte is 25-mM sodium acetate in 100-mM Phosphate Buffer Solution (PBS) with 1,600 mg NaCl, 380 mg NH_4Cl, 5 mg EDTA, 30 mg $MgSO_4 \cdot 7H_2O$, 5 mg $MnSO_4 \cdot H_2O$, 10 mg NaCl, 1 mg $Co(NO_3)_2$, 1 mg $CaCl_2$, 0.001 mg $ZnSO_4 \cdot 7H_2O$, 0.001 mg $ZnSO_4 \cdot 7H_2O$, 0.1 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg $AlK(SO_4)_2$, 0.1 mg H_3BO_3, 0.1 mg $Na_2MoO_4 \cdot 2H_2O$, 0.1 mg Na_2SeO_3, 0.1 mg $Na_2WO_4 \cdot 2H_2O$, 0.2 mg $NiCl_2 \cdot 6H_2O$, and 1 mg $FeSO_4 \cdot 7H_2O$ (per liter of deionized water). For start-up process, inoculum and anolyte are mixed with 1:1 volume ratio as anolyte. The catholyte is 50-mM potassium ferricyanide in 100-mM PBS. Anolyte and catholyte were supplied to the MFC through a syringe pump.

Stability and Electrochemical Characterization

The potential stability of the reference electrode was measured against a commercially available Ag/AgCl in 3M NaCl reference electrode. The voltage profiles were recorded by a data acquisition system (DAQ/68). Then polarization curves were obtained by recording voltage across a series of resistors connected between the anode and cathode, ranging from 148 Ω to 1 MΩ. Anode and cathode potentials were also measured against a commercially available Ag/AgCl in 3M NaCl electrode.

Acetate, Bicarbonate and Proton Quantification

Effluent from the reference electrode was collected. For acetate quantification, the acetate colorimetric assay kit was implemented (Sigma Aldrich) by following the instruction. The acetate concentration was calculated by substituting into the linearly fitted absorption data the standard. Carbon dioxide enzymatic assay kit (Bioo Scientific) was implemented for the bicarbonate measurement by following the instructions. The bicarbonate concentration were calculated by substituting into the linearly fitted absorption data the standard. The proton concentration was measured by a pH meter. Concentration of protons is calculated as $[H^+]=1/10^{pH}$ mole/L.

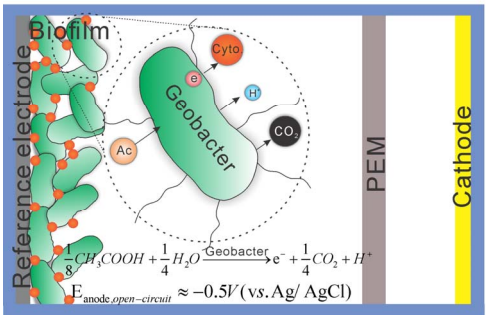

Figure 2. The operation principle of the bio-inspired reference electrode. A layer of Geobacter sulfurreducens -enriched biofilm forms on the electrode, which serves as catalyst to break down acetate and the electrochemical potential for the reaction. The biochemical reaction sets the electrode potential to be very stable ~ -0.5 V versus a commercially available reference electrode, Ag/AgCl in 3M NaCl.

RESULTS AND DISCUSSION

Operation Principle

A layer of *Geobacter* enriched biofilm forms on the anode. The *Geobacter* is an exoelectrogen which can transfer electrons outside its outer membrane to complete its respiration process. In our biologically-inspired reference electrode, it serves as catalyst to break down the acetate in the anolyte to generate electrons, protons, and carbon dioxide, as shown in Equation 1[11]. This is an electrochemical reaction and has an electrochemical potential of ~-0.5V versus Ag/AgCl in 3M NaCl, as illustrated in equation 1 and Figure 2 [12, 13]. Open circuiting the reference electrode forces exoelectrogens on the electrode unable to transfer electrons to the electrode to complete the respiration process will set the reference potential to be the open circuit potential of the anolyte, sodium acetate, which is ~ -0.5 V versus the Ag/AgCl in 3M NaCl. Thus, after growing the *Geobacter* enriched biofilm, we implement the biofilm to provide the stable reference electrode.

$$\frac{1}{8}CH_3COOH+\frac{1}{4}H_2O \xrightarrow{\text{Geobacter}} e^- + \frac{1}{4}CO_2 + H^+$$

$$E_{anode,open-circuit} \approx -0.5V(vs.Ag/AgCl) \quad (1)$$

The operation principle is firstly verified by the Nernst equation. The Nernst equation for equation 1 is written as

$$E=E_0 - \frac{RT}{8F}\ln\left(\frac{CH_3COO^-}{[HCO_3^-]^2[H^+]^9}\right) \quad (2)$$

Where E is the electrochemical potential of the reference electrode, E^0 is the standard cell electrochemical potential for equation 1, R is the universal gas constant, T is the absolute temperature, F is the Faraday's constant. $[CH^3COO^-]$, $[HCO_3^-]$ and $[H^+]$ are the concentration of aceate, bicarbonate and protons, respectively. The pH of the effluent to be 6.84, and thus $[H^+]=1.445\times10^{-7}$ mole/L. The concentrations of acetate and bicarbonate are calculated by the optical absorption approach depicted in the Materials and Methods section to be 19.288 mM and 9.34 mM, respectively. Comparing the electrochemical potential by Nernst equation of -0.495V vs. Ag/AgCl in 3M NaCl with the electrochemical potential of the reference electrode in Figure 3 of -0.460±0.004 V vs.

Ag/AgCl in 3M NaCl, we can verify the operation principle of the reference electrode.

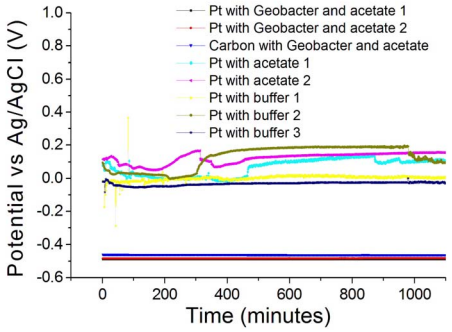

Figure 3. Proof of the operation principle of the biologically-inspired reference electrode. When Geobacter inoculum is present, the electrode potential is almost fixed at approximately -0.5V versus Ag/AgCl in 3M NaCl for both Pt and carbon electrodes, while when no Geobacter inoculum exists the electrode potential varies greatly, and it cannot operate as a reference electrode.

The operation principle of the biologically-inspired reference electrode was verified by comparing the potential of the reference electrode with a group of controls, as depicted in Figure 3. When *Geobacter* enriched biofilm forms on the reference electrode, either Pt or carbon, stable potential forms at approximately -0.5V versus Ag/AgCl in 3M NaCl. In contrast, when biofilm do not form on the anode, the potential of the reference electrode fluctuate significantly, thus the *Geobacter* enriched biofilm is critical for the operation of the biologically-inspired reference electrode. Furthermore, we used a strong acidic solution to bleach the *Geobacter*-covered electrode, and it showed poor potential stability, which suggest the micro-organisms are essential for the stable potential over the time.

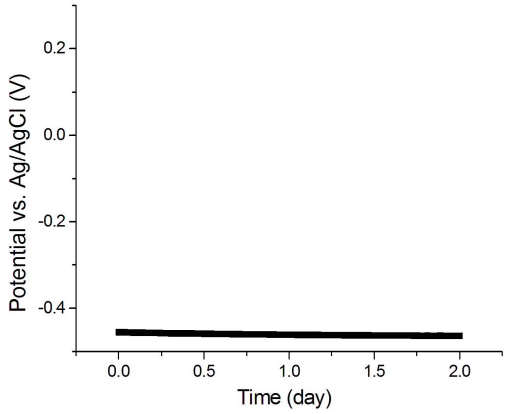

Figure 4. Long-term stability of the biologically-inspired reference electrode potential (vs. Ag/AgCl in 3M NaCl) for more than 1 week. The electrode had a stable potential of -0.465 V with a stability of ±4.2 mV/day.

Long-term stability

The long-term stability of the biologically-inspired reference electrode is characterized. As illustrated in Figure 4, it showed a stable potential of -0.460 V with a stability of ±4.2 mV/day for two days. The stability period is 116 and 11 fold higher, respectively, compared with Polk et al. 2006 and Safari et al. 2011[9, 10].

Electrochemical characterization

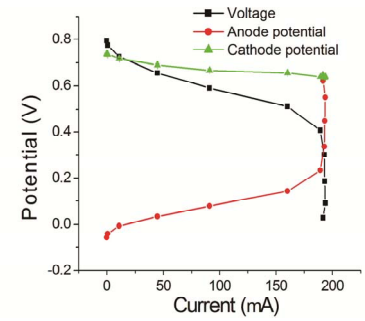

Figure 5. The polarization curve of the MEMS MFC, which includes the output voltage, anode and cathode potentials.

The biologically-inspired reference electrode is integrated in a MEMS microbial fuel cell (MFC). A thin film Au electrode is applied as anode. The polarization curve of the MEMS MFC is shown in Figure 5. The polarization curve includes the output voltage of the MFC, the anode and cathode potentials. The biologically-inspired reference electrode enables the measurement of both the anode and cathode potentials, which have been difficult for previously reported MEMS MFCs [14-16]. As shown in Figure 5, as current increases, the cathode potential do not show a remarkable change, while the anode potential changes significantly. A significant change shows especially in the concentration region where the anode potential shows a steep increase while the current of the MEMS MFC holds almost constant. This is in agreement with prior arts that the current of the anode is limiting for the operation of MFCs[4, 17, 18]. As far as the authors are aware, this is the first time to confirm the anode to be the limiting factor in MEMS MFCs.

CONCLUSION

In summary, inspired by the possibility of controlling the respiration of bacteria, this paper presents a novel reference electrode having ±4.2 mV/day stability for two days. The operation principle and long-term stability of the biologically-inspired reference electrode are studied. The potential stability is due to the *Geobacter* enriched biofilm on the reference electrode, which forms an electrochemical potential of ~ -0.5 V versus the Ag/AgCl in 3M NaCl, which is confirmed by colorimetric analysis. The biologically-inspired reference electrode demonstrates stability of ±4.2 mV/day for two days. The reference electrode integrated inside a MEMS MFC. Polarization and voltammetry curves are both obtained by the biologically-inspired reference electrode, which confirms the anode to be the limiting factor for the MEMS MFC for the first time and report the voltammetry curve for the MEMS MFC for the first time.

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