A BIO-INSPIRED REFERENCE ELECTRODE: REGULATING THE RESPIRATION OF MICRO-ORGANISMS

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ABSTRACT

Inspired by the possibility of controlling the respiration of micro-organisms, we present a bio-inspired reference electrode, demonstrating a stable potential for extended period of time. The bio-inspired reference electrode, made of platinum, sits in a microfluidic chamber for growing specific micro-organisms, *Geobacter sulfurreducens*, which is an exoelectrogen capable of transferring electrons outside its outer membrane to complete its respiration process. Open circuiting the reference electrode forces exoelectrogens on the electrode unable to transfer electrons to the electrode to complete the respiration process. This 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. The bio-inspired reference electrode maintained its potential stability of ± 1.07 mV/day for over a week. The bio-inspired reference electrode was integrated in a MEMS microbial fuel cell (MFC) to characterize its electrochemical characteristics, including polarization, voltammetry, and electrochemical impedance spectroscopy.

INTRODUCTION

Bio-inspired devices and systems have been an active area of research, including bio-inspired digital cameras [1], bio-inspired artificial lungs [2], bio-inspired camouflage and display for soft machines [3] and bio-inspired supercapacitors [4], which reported not only novel technological advancements but also significant performance enhancement.

MEMS and lab-on-a-chip have been on active research through successful miniaturization of sensors, actuators, and systems [5, 6]. Reference electrodes, which offer maintaining a stable potential, have been widely used in electrochemistry, such as fuel cells and electrochemical sensors [7, 8]. As conventional references, i.e., Ag/AgCl, are too bulky to be integrated in MEMS and lab-on-a-chip setups, researchers have explored miniaturizing reference electrodes in the past. Despite the extensive studies, a microfabricated reference electrode in MEMS / 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.* electroplated Ag/AgCl thin films to be a reference electrode, yet marked rather poor stability, ± 2.88 mV/day for merely 1.4 hours [9]. Another attempt deposited silver chloride nanosheets on silver wire, however, again the reported stability, ± 3.2 mV/day for 15 hours, is far from practical use, demanding substantial improvement [10]. Reference electrodes need to provide a stable potential for an extended period, i.e., several days, when being implemented in electrochemical applications such as microbial electrochemical techniques, environmental monitoring, *etc.*

The aforementioned microfabricated reference electrodes, unfortunately, fall shorts for the demand. The bulky conventional reference electrode not only forces the overall devices / systems to occupy a large footprint, but also often cannot be used in MEMS and lab-on-a-chip. This directly motivates our work: studying a miniaturized reference electrode capable of maintaining its potential for an extended period, over a week.

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 (insets: scanning electron microscopy (SEM) image of the Pt reference electrode with *Geobacter* biofilm formed on top, scale bar: 1 μ m).

MATERIALS AND METHODS

Reference electrode and cathode fabrication

The bio-inspired reference electrode was fabricated by patterning a thin layer of Ti/Pt (20nm/100nm) by lift-off to form an electrode. First, six through holes were drilled on a glass slide (46 mm $\times$ 26 mm $\times$ 1 mm, VWR): one as inlet, one as outlet and the rest four as screw holes. Then the glass slide was cleaned by piranha solution (concentrated H₂SO₄ and 30% H₂O₂, 3:1 volume ratio). Subsequently a layer of photoresist (AZ 4330, 4 μ m thick) was patterned on the glass slide, and a layer of Ti/Pt (20/100 nm) was deposited using an electron beam evaporator. The reference electrode was patterned by lifting Ti/Pt off in acetone. The optical microscopy and cross-sectional images of the reference electrode is shown in Figure 1 (a, b). The cathode in this

work was made of gold. A layer of Cr/Au (20/200 nm) was deposited using a magnetron sputter on a glass slide (46 mm × 26 mm × 1 mm, VWR).

Device assembly

The reference electrode, cathode, proton exchange membrane (PEM, Nafion 117), silicone gaskets (250 μm thick, Fuel Store Inc.), nanoports (10-32 Coned assembly, IDEX Health & Science), bolts and nuts were assembled to build the MEMS device to evaluate the bio-inspired reference electrode for the electrochemical characterization. Nanoports were glued to the inlet and outlet of reference electrode and cathode on two glass slides. Then, two rectangular 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 25 μL. Finally, a rectangular PEM was cut, and PEM and silicone gaskets were sandwiched between the two glass slides having the reference electrode and cathode to complete the device.

Geobacter sulfurreducens enriched biofilm formation

Geobacter sulfurreducens enriched bacterial community, originally from anaerobic-digestion sludge, was used as inoculum to form a biofilm for the bio-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 NH₄Cl, 5 mg EDTA, 30 mg MgSO₄·7H₂O, 5 mg MnSO₄·H₂O, 10 mg NaCl, 1 mg Co(NO₃)₂, 1 mg CaCl₂, 0.001 mg ZnSO₄·7H₂O, 0.001 mg ZnSO₄·7H₂O, 0.1 mg CuSO₄·5H₂O, 0.1 mg AlK(SO₄)₂, 0.1 mg H₃BO₃, 0.1 mg Na₂MoO₄·2H₂O, 0.1 mg Na₂SeO₃, 0.1 mg Na₂WO₄·2H₂O, 0.2 mg NiCl₂·6H₂O, and 1 mg FeSO₄·7H₂O (per liter of deionized water) (pH 7.8 ± 0.2). For start-up process, inoculum and anolyte were mixed with 1:1 volume ratio. The catholyte is 50-mM potassium ferricyanide in 100-mM PBS (pH 7.4). Anolyte and catholyte were supplied to the MFC through a syringe pump (PHD 2000, Harvard Apparatus).

Stability and Electrochemical characterization

The stability of the reference electrode was evaluated with a commercially available Ag/AgCl in 3M NaCl reference electrode (BASi). A data acquisition system (DAQ/68, National Instrument) recorded the voltage profiles. 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. The DAQ recorded anode and cathode potentials independently. A potentiostat (Gamry Instruments) was used to perform electrochemical impedance spectroscopy measurements, by sweeping the frequency between 100 mHz and 100 kHz with a 30 mV_{pp} ac voltage excitation. Gamry Analyst software fit the data from the measurements.

RESULTS AND DISCUSSION

Operation principle

Figure 1(c) shows the bio-inspired reference electrode enclosed in a 25 μL microfluidic chamber. Layers of *Geobacter sulfurreducens* enriched biofilm form on the anode in the 25 μL reactor. The *Geobacter sulfurreducens* is an exoelectrogen capable of transferring electrons outside its outer membrane to complete its respiration process. In our application it serves as catalyst to break down the acetate in the anolyte to generate electrons, protons, and carbon dioxide, as depicted in Equation 1 [11].

Figure 2. The operation principle of the bio-inspired reference electrode. Layers of *Geobacter sulfurreducens*-enriched biofilm form on the electrode, which serves as catalyst to break down acetate and sets 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.

Figure 3. Verification of the operation principle of the bio-inspired reference electrode. When *Geobacter* inoculum is present, the reference electrode potential is almost constant at approximately -0.5V versus Ag/AgCl in 3M NaCl (for both Pt and carbon electrodes), while when no *Geobacter* inoculum exists the potential varies greatly.

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. Consequently the open circuit 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, the *Geobacter* enriched biofilm grown electrode may serve as the stable reference.

The bio-inspired reference electrode was evaluated by comparing the potential of the reference electrode with a group of controls, as depicted in Figure 3. When *Geobacter sulfurreducens*-enriched biofilm forms on the reference electrode, either Pt or carbon, stable potential establishes at approximately -0.5 V versus Ag/AgCl in 3M NaCl. In contrast, when no biofilm exists on the anode, the potential of the electrode fluctuates significantly, with a stability of mere ± 42 mV/day. This verifies the *Geobacter sulfurreducens*-enriched biofilm is critical for the operation of the bio-inspired reference electrode. Furthermore, we used a strong acidic solution to bleach the *Geobacter*-covered electrode, showing poor potential stability of ± 37 mV/day, suggesting the micro-organisms deliver key features of stable potential over the time.

Long-term stability

Figure 4. Long-term stability of the bio-inspired reference electrode potential (vs. Ag/AgCl in 3M NaCl) for > 1 week. The electrode maintained a stable potential of -0.465 V with a stability of ± 1.07 mV/day; insets: a comparison of stability and period of the bio-inspired reference electrode with prior art on microfabricated reference electrodes, showing the bio-inspired reference electrode provides substantially more stable potential and longer stability period over others.

The long-term stability of the bio-inspired reference electrode was evaluated as well. As illustrated in Figure 4, the bio-inspired reference electrode presents a very stable potential of -0.465 V with a stability of ± 1.07 mV/day for over a week. The stability of the reference electrode is 2.7 and 3.0 fold higher respectively, while the stability period is 116 and 11 fold longer, respectively, over prior work [9, 10]. Both the stability itself and the stability period support the excellent performance of the bio-inspired reference electrode.

Electrochemical characterization

The bio-inspired reference electrode was integrated in a MEMS microbial fuel cell (MFC) to explore the usefulness. A thin film Cr/Au electrode served as anode. Figure 5(a)

shows the polarization curve of the MEMS MFC. The polarization curve includes the voltage across the load as well as anode and cathode potentials, with respect to the reference potential. The curve shows three sections: the activation region, the linear region and the concentration region. The integrated bio-inspired reference electrode allows the independent measurement of the anode and cathode potentials, which has been considered as a significant challenge in the previously reported MEMS MFCs [14-16]. As the current increases (the external load decreases), the cathode potential shows little change, reducing from 0.74 V to 0.64 V, while the anode potential changes significantly, increasing from -0.06 V to 0.62 V. The significant anode potential change becomes vivid, from 0.23 V to 0.62 V, in the concentration region where the current remains almost unchanged. This agrees well with prior studies that the current generation capability of the anode limits the operation of MFCs [4]. The linear fit of the linear region of the polarization curve yields an internal resistance of $1,640 \pm 148 \Omega$. Figure 5(b) shows the quasi-voltammetry curve. The voltammetry curve follows a typical sigmoid shape and the mid-potential for the quasi-voltammetry curve is 0.08 V versus the bio-inspired reference electrode, which is also in good agreement with prior arts [4, 17].

Figure 5. Electrochemical characterization of a MEMS Microbial Fuel Cell (MFC) using the bio-inspired reference electrode, integrated inside the anode chamber: (a) polarization curve, which includes the potentials across the load as well as anode and cathode potentials, with respect to the reference potential; the polarization curve shows three sections, the activation region, the linear region and the concentration region; (b) quasi-voltammetry curve; both curves are well in agreement with prior arts. The mid-potential for the quasi-voltammetry curve is 0.08 V versus the bio-inspired reference electrode, which is also in good agreement with prior

arts.

Figure 6. Electrochemical impedance spectroscopy characterization of the MEMS MFC, integrated with the bio-inspired reference electrode. The total internal resistance, 1,891 Ω agrees with the internal resistance by polarization curve.

Figure 6 shows electrochemical impedance spectroscopy characterization of the MEMS MFC, integrated with the bio-inspired reference electrode. The total ohmic resistance of the MEMS MFC was measured to be 1,891 Ω , agreeing well with the internal resistance obtained by the linear fitting of the polarization curve.

CONCLUSION

In summary, inspired by the possibility of controlling the respiration of micro-organisms, this study presents a novel reference electrode having ± 1.07 mV/day stability over one week. This work explores the operation principle of the bio-inspired reference electrode using *Geobacter sulfurreducens* enriched biofilm on the reference electrode. The long-term stability of the bio-inspired reference electrode was evaluated using a series of controls as well. The long-term stability benefits from the *Geobacter sulfurreducens* enriched biofilm on the reference electrode, which establishes a stable electrochemical potential of ~ -0.5 V versus the Ag/AgCl in 3M NaCl. The bio-inspired reference electrode was integrated in a MEMS MFC shows standard electrochemical measurements, including polarization, voltammetry and electrochemical impedance spectroscopy. Those data agree well with prior arts which use a bulky Ag/AgCl reference electrode, demonstrating the bio-inspired electrode indeed serves as an alternative stable reference.

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