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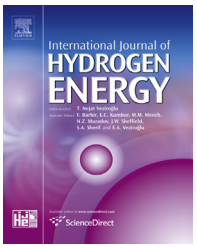

Short Communication

The effect of biofilm thickness on electrochemical activity of *Geobacter sulfurreducens*

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ABSTRACT

It is widely believed that the high electrochemical performance of *Geobacter sulfurreducens* relies on its thick and conductive biofilms in bioelectrochemical systems. However, in this study, the *G. sulfurreducens* biofilm reached the highest electrochemical activity with a biofilm thickness of ~20 μm, and then the electrochemical activity decreased with increasing thickness until the biofilm growth ceased at a thickness of ~45 μm. The electrochemical analysis and the metabolic spatial variability showed that in the first 5 cycles the live cells grew fast, which led to a rapid drop of charge transfer resistance and further contributed to high current generation, however, from cycle 5 to 12, a great many inactive cells accumulated in the inner layer of biofilm, which resulted in high diffusion resistance. Thus, although the *G. sulfurreducens* can always form thick biofilms, its highest electrochemical activity reached at a much thinner thickness, suggesting that the live-cell mass rather than the biofilm thickness is responsible for the high current generation.

Introduction

Bioelectrochemical system (BES) is an emerging technology for many potential applications on sustainable energy and environmental Protection, including electricity generation, biohydrogen production, desalination, wastewater treatment and so on [9,12,19–21]. In most bioanode BESs, anode microorganisms function as catalysts to extracellular transfer electrons (EET) into electrode [12,13]. Without the addition of artificial electron shuttles, three EET mechanisms have been extensively studied: (a) electron transfer via cell self-produced electron shuttles, such as *Pseudomonas aeruginosa* and *Shewanella oneidensis* [22,31]; (b) short-range electron transfer through redox-active proteins by microorganisms in close association with the electrode surface, such as *S. oneidensis* and *Therminocola potens* [10,32]; (c) long-range electron transport through conductive and thick biofilms, such as *Geobacter sulfurreducens* and *Geobacter anodireducens* [16,29]. It is generally accepted that the mode of long-range electron transport is the best for current generation, which permits multiple layers of cells at distances of tens of microns from

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the anode to donate electrons to generate current in BESs [6,14,23]. For example, the model exoelectrogenic microbe, *G. sulfurreducens*, is capable of forming a thick and conductive biofilm on an anode surface and is able to produce a current comparable to those of mixed cultures [15,18]. Thus, as pointed out in lots of studies, the high electrochemical activity of *G. sulfurreducens* is considered to be positively correlated with the biofilm thickness or the total biomass [6,7,13].

The operation currents of *G. sulfurreducens* and mixed cultures in numerous BESs reached stable in initial several batch cycles (<3 cycles or <7 days) with newborn and thin biofilms, and the further growth of biofilms cannot improve the current density [2,4,5,34]. Moreover, the electrochemical activity of the aged biofilm would decrease due to the large diffusion resistance in the thick biofilm [24]. This indicated that although the *G. sulfurreducens* cells long-distance away from the anode can transfer electrons, its biofilm biomass did not always positively affect the current generation, and the ideal biofilm thickness for current generation may much thinner than that its biofilm can achieve. Thus, the investigation of biofilm thickness effects on electrochemical performance is needed to optimize the BES performance. In this study, the electrochemical activity, metabolic spatial variability and biomass of *G. sulfurreducens* biofilms were simultaneously and quantitatively analyzed during the whole growing process.

Materials and methods

Mini-BES construction and operation

Tens of mini-BESs were constructed and operated as previously described [3,8,26,30]. Anodes were 1.5 cm × 1 cm × 0.3 cm (4.5 cm^2 surface area) polished graphite plates (Grade GM-10; GraphiteStore.com Inc.). The cathodes were 1.5 cm × 1 cm stainless steel mesh (Type 304, mesh size 90 × 90; McMaster-Carr) [1,35]. An added voltage of 0.7 V was applied here using a power supply. The initial inoculum was the effluent from one mini-BES that was inoculated with *G. sulfurreducens* PCA (ATCC 51573) operated one week period as previously described [26]. BESs were operated with the 50 mM phosphate buffer solution (PBS) nutrient medium contained (per liter): 1 g acetate, 2.45 g $NaH_2PO_4 \cdot H_2O$, 4.58 g Na_2HPO_4, 0.31 g NH_4Cl, 0.13 g KCl, 12.5 mL metal salts and 5 mL vitamins (pH = 7; conductivity = 7.5 mS/cm). All reactors were operated with batch mode at 30 °C. The cycle time of the mini-BES is one day, except for the first cycle (2–3 days). Standard anaerobic techniques were used throughout all procedures [3]. The medium was sparged with N_2 and all mini-BESs were filled with 100% N_2 in the headspace before being inoculated. The sterile syringe was used to replace the medium at the end of each batch cycle. Based on the current generation and the biofilm growth, four representative growing phases of biofilms were analyzed: (a) initial phase: current recorded at 0.1 mA (initial current) in the cycle 1 and at the end of the cycle 1; (b) fast cell accumulation phase: at the end of cycle 2 and 3; (c) highest electrochemical activity phase: at the end of cycle 5; (d) mature phase: at the end of cycle 12 and 30. For each testing time-point, three mini-BESs would be used to examine the metabolic spatial variability, protein and electrochemical property respectively.

Electrochemical analysis

Linear sweep voltammetry (LSV), first derivative LSV and electrochemical impedance spectroscopy (EIS) were examined using a potentiostat (CHI660D; Chenhua, China; EC-Lab V10.02 software) as previously described [26,27]. The anode was the working electrode, the cathode was the counter electrode, and an Ag/AgCl electrode (+200 mV vs. a standard hydrogen electrode, SHE) was used as the reference electrode. For LSV analyses, the reactors were scanned from −0.50 to +0.30 V at a rate of 1 mV/s. EIS was conducted at a set potential equal to the mid-point potentials (−0.15 V) obtained in first derivative LSVs, over a frequency range of 200 kHz to 10 mHz, with a sinusoidal perturbation of 5 mV amplitude. The EIS spectra with two circles were fitted into the equivalent circuit containing a solution resistance (R_s), two charge transfer resistances (R_{ct1} and R_{ct2}), a diffusion resistance (R_d), and double layer capacitance (Q) (Fig. S1) [26]. All electrode potential values are reported here versus SHE.

Biofilm growth and metabolic spatial variability

Biofilm growth was monitored based on the protein content extracted from the entire anode using a Bicinchoninic acid protein assay kit. The metabolic spatial variability of biofilms was observed by using LIVE/DEAD BacLight™ Bacterial Viability Kit (Invitrogen, CA) and confocal laser scanning microscope (CLSM, LSM710 NLO, ZEISS) with a water objective (LD LCI Plan-Apochromat 25/0.8 Imm Korr DIC), and the three-dimensional biofilm metabolic-status (z-stack) was reconstructed and analyzed by the software of ZEN 2009 Light Edition.

Results and discussion

Current generation and biofilm growth

The *G. sulfurreducens* immediately produced current after inoculation and reached its highest operation current density (recorded in batch cycle) during the cycle 5 to 9, after then the current began to decrease, and finally reached stable from cycle 12 to 30 (Fig. 1a). In the first cycle, the operation current density was 1.23 ± 0.01 A/m^2, which already reached 56% of the highest operation current density (2.21 ± 0.06 A/m^2, from cycle 5 to 9) and 72% of the stable operation current density (1.71 ± 0.06 A/m^2, from cycle 12 to 30) (Fig. 1a). However, the simultaneous protein content of the biofilm in the cycle 1 was only 0.30 g/m^2, which was 21% of that in the cycle 5 (1.39 g/m^2) and 8% of that in the cycle 30 (3.62 g/m^2) (Fig. 1). The maximum current densities recorded in LSVs were consistent with the operation currents (Fig. 1). The *G. sulfurreducens* achieved the highest maximum current density of 3.76 A/m^2 in the cycle 5, with 38% biomass of the final biomass in the cycle 30. This demonstrated that high current can be generated by the relatively thin biofilm of *G. sulfurreducens*, and suggested that biofilm thickness affected the electrochemical activity in

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