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Microbial activity influences electrical conductivity of biofilm anode

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Abstract

This study assessed the conductivity of a *Geobacter*-enriched biofilm anode in a microbial electrochemical cell (MxC) equipped with two gold anodes (25 mM acetate medium), as different proton gradients were built throughout the biofilm. There was no pH gradient across the biofilm anode at 100 mM phosphate buffer (current density 2.38 A/m²) and biofilm conductivity (K_{bio}) was as high as 0.87 mS/cm. In comparison, an inner biofilm became acidic at 2.5 mM phosphate buffer in which dead cells were accumulated at ~80 μm of the inner biofilm anode. At this low phosphate buffer, K_{bio} significantly decreased by 0.27 mS/cm, together with declined current density of 0.64 A/m². This work demonstrates that biofilm conductivity depends on the composition of live and dead cells in the conductive biofilm anode.

Graphical Abstract

Keywords

Acidic biofilm; Biofilm anode; Biofilm conductivity; Extracellular electron transfer; Microbial electrochemical cell

1. Introduction

Extracellular electron transfer (EET) is unique for anode-respiring bacteria (ARB) to balance intracellular reducing power using solid electron sinks (Torres et al., 2010, Lee et al., 2009, Lee et al., 2016, Rotaru et al., 2015). Microbial electrochemical cells (MxCs) employing ARB as an anode catalyst are very promising, since they are able to recover value-added products from organic waste and wastewater (Logan et al., 2015, Feng et al., 2016, Dhar et al., 2016a). High current density is essential for catalyzing MxC deployment in field, and thus understanding EET kinetics can be important for achieving high current density in MxCs. Among several EET mechanisms, conductive EET allows biofilm anodes to generate high current density in MxCs (Torres et al., 2010, Torres et al., 2008a, Lee et al., 2016, Marcus et al., 2007, Renslow et al., 2013). Electron conduction of EET can occur through electrically conductive pili (Reguera et al., 2005, Malvankar et al., 2011, Xiao et al., 2016), extracellular cofactors (Snider et al., 2012, Strycharz-Glaven and Tender, 2012, Yates et al., 2016, Phan et al., 2016), or its combination (Lee et al., 2016). The literature reported that the electrical conductivity of a *Geobacter sulfurreducens* biofilm anode was as high as ~ 5 mS/cm (Malvankar et al., 2012), and the conductivity of *Geobacter*'s pili is even higher (Adhikari et al., 2016). For *Geobacter*-enriched mixed culture biofilm anodes, high biofilm conductivity (K_{bio}) of 0.96–2.44 mS/cm was also reported (Lee et al., 2016, Dhar et al., 2016b). The literature has suggested EET would occur through electrically conductive pili in biofilm anodes having high K_{bio} (Malvankar et al., 2011, Malvankar et al., 2012, Xiao et al., 2016, Adhikari et al., 2016). In comparison, a low range of K_{bio} (0.0003–60 $\mu\text{S}/\text{cm}$) was observed for *Geobacter sulfurreducens* biofilm anodes when redox conduction via extracellular cofactors would be responsible for conductive EET (Yates et al., 2016, Phan et al., 2016). These K_{bio} values are several orders of magnitude lower than the highest K_{bio} in a *Geobacter* pure culture or enriched biofilm anode where electrically conductive pili would play an important role of EET. Unfortunately, there are no clear mechanisms accounting for substantial K_{bio} difference in electrically conductive biofilm anodes, in which two different EET pathways would occur. Conductive EET mechanisms seem more complicated than early research suggested (Torres et al., 2010, Reguera et al., 2005, Malvankar et al., 2011, Snider et al., 2012), and thus it is challenging to assess dominant EET mechanism for conductive biofilm anodes. However, modeling approach based on biofilm conduction (i.e., the Nernst-Monod equation) suggests that K_{bio} would be higher than 0.5 mS/cm in biofilm anodes generating high current density without substantial energy losses (Marcus et al., 2007, Renslow et al., 2013), which emphasizes K_{bio} significance to success of MxCs as resource recovery wastewater treatment technologies.

Anode respiration allows ARB only to transfer electrons to the anode, accumulating substantial protons in biofilms. This unique respiration can acidify biofilm anodes (Torres et al., 2008b, Marcus et al., 2010, Marcus et al., 2011, Franks et al., 2009). The challenge is

that ARB's metabolism is seriously inhibited at acidic pH (Franks et al., 2009, Kim and Lee, 2010, Patil et al., 2011). ARB (e.g., *Geobacter*) grow well at neutral pH, although some alkaliphilic ARB (e.g., *Geoalkalibacter* spp.) were identified (Pierra et al., 2015; Yoho et al., 2015). For instance, the growth rate of *Geobacter sulfurreducens* at pH 6 was decreased to 80% over pH 7 (Franks et al., 2009). Literature also reported considerable decrease of current density in MxCs when bulk pH or local biofilm anodes were acidic (Franks et al., 2009, Kim and Lee, 2010, Patil et al., 2011). It is apparent that acidic pH seriously inhibits electron transfer rate from donor substrate to the anode in biofilm anodes.

Despite the significance of acidic pH for electron transfer rate in biofilm anodes, understanding of EET kinetics at acidic pH is very limited. Malvankar et al. (2012) reported the decrease of K_{bio} (from 5 to 0.25 mS/cm) and current density (from 10 to 2 A/m²) for a thick biofilm (130 μm) in which mass transfer limitations of protons or substrate might decrease K_{bio} and current density. Interestingly, the conductive pili of *Geobacter sulfurreducens* showed higher electrical conductivity at pH 2 than neutral and alkaline pHs (Malvankar et al., 2011, Adhikari et al., 2016), which is opposite to K_{bio} reduction in acidic pH (Malvankar et al., 2012). Understanding of EET kinetics at acidic pH can be very important for MxC deployment in field where acidic biofilms can be built due to relatively low buffer conditions in wastewater. However, there are no clear, detailed information on K_{bio} and EET kinetics at acidic biofilm anodes.

In this study, we evaluated the change of biofilm conductivity in a steady-state biofilm anode in which different proton gradients were built using three phosphate buffer concentrations. First, current density, biofilm thickness, and proton gradients were quantified for individual phosphate conditions. Second, the metabolic activity of ARB within biofilm anodes was qualitatively compared between low and high phosphate buffer using confocal laser scanning microscopy (CLSM). Finally, we experimentally measured K_{bio} and half-saturation anode potential (E_{KA}) for the biofilm at the three phosphate buffer concentrations and discussed the implication of reduced K_{bio} at acidic pH.

2. Materials and methods

2.1. MxC configuration and operation

Three dual-chamber MxCs were constructed with plexiglass: two biotic and one abiotic MxCs (see Fig. 1(a)). The abiotic MxC identical to the two biotic MxCs was used for measuring ionic conductance to quantify intrinsic biofilm conductance. Two gold electrodes (width 9.5 mm $\times$ length 15 mm $\times$ thickness 10 μm) on a glass base with a non-conductive gap of 50 μm were designed as anodes to measure biofilm conductivity (Dhar et al., 2016b), and the total geometric surface area of the anodes was 2.85 cm² (see Fig. 1(b)). A porous graphite plate (Isomolded Graphite Plate 203101, Fuel Cell Earth, USA) was used as the cathode and anion exchange membrane (AMI-7001, Membranes International Inc., USA) was inserted between the anode and the cathode chambers as a separator. The working volumes of both chambers were 15 mL. A reference electrode (Ag/AgCl reference electrode, MF-2052, Bioanalytical System Inc., USA) was placed within less than 1 cm distance from the anodes to fix anode potential (E_{anode}) during experiments.

Two MxCs were inoculated with a biofilm anode collected from a mother MxC that had been operated with acetate medium (25 mM acetate) for over one year. The anode chambers were filled with 25 mM acetate medium (100 mM phosphate buffer, pH 7.25–7.4), and the cathode chambers were filled with tap water, producing H₂ gas; the literature provides the composition of acetate medium (Dhar et al., 2013). We fixed anode potentials (E_{anode}) at –0.2 V against standard hydrogen electrode (SHE) using a potentiostat (BioLogic, VSP, Gamble Technologies, Canada). The anode chambers were sparged with ultra-pure nitrogen (99.999%) for 5 min before operation of the MxCs. Current was recorded at every 2 min using EC-Lab for windows v 10.32 software in a personal computer connected to the potentiostat. After operating the MxCs for 5 days in batch mode (the maximum current density $\sim 0.7 \text{ A/m}^2$), we continuously fed acetate medium to the MxCs at a flow rate of 5.8 mL/h using a peristaltic pump (Master Flex® L/S digital drive, Model 7523–80, Cole-Parmer, Canada). Hydraulic residence time (HRT) in the anode chambers was kept at 2.6 h, leading to substrate non-limiting conditions for anode-respiring bacteria (ARB). Phosphate buffer concentration in acetate medium was stepwise decreased from 100 mM to 2.5 mM (pH 7.2–7.4) to create different pH gradients throughout the biofilm anode of a MxC ($\text{MxC}_{\text{buffer}}$). At the steady-state biofilm anode for current density and effluent acetate concentration at each buffer concentration in the $\text{MxC}_{\text{buffer}}$, pH gradients throughout the biofilm anode, biofilm conductivity (K_{bio}), half-saturation anode potential (E_{KA}), and biofilm thickness (L_f) were measured. To mitigate the changes of biofilm community structure and biofilm thickness at 50 and 2.5 mM phosphate buffer, we completed the experiments for the low phosphate buffer concentrations in ~ 2 weeks.

2.2. Biofilm conductivity

The conductivity of the biofilm anode grown at different phosphate buffer concentrations in the $\text{MxC}_{\text{buffer}}$ was measured using the two-probe measurement method (Malvankar et al., 2011, Lee et al., 2016, Dhar et al., 2016b). For the biofilm conductance measurement, the gold anodes and the cathode were disconnected temporarily (open circuit mode). Then, a linear sweeping voltage of 0–0.05 V in a step of 0.025 V was applied across two gold electrodes using a source meter (Keithley 2400, Keithley Instruments, Inc., USA), and the current was recorded in 1 min for each voltage. The voltage ramp was applied in 4–5 cycles until a steady-state current-voltage (I-V) response was obtained; we confirmed negligible effects of anode potential for the steady state I-V responses (Dhar et al., 2016b). Observed biofilm conductance ($G_{\text{Biofilm (obs)}}$, mS) was calculated from steady-state I-V curves (Dhar et al., 2016b, Lee et al., 2016). Ionic current can contribute to $G_{\text{Biofilm (obs)}}$ values, and therefore the ionic conductance (G_{control} , mS) was measured with the abiotic MxC (control) using the acetate medium and MxC effluent as electrolytes. Intrinsic biofilm conductance ($G_{\text{Biofilm}} = G_{\text{Biofilm (obs)}} - G_{\text{control}}$, mS) was quantified with Eq. (1) (Kankare and Kupila, 1992).

$$K_{\text{bio}} = \frac{G_{\text{Biofilm}} \frac{\pi}{L}}{\ln\left(\frac{8L_f}{\pi a}\right)} \quad (1)$$

where, G_{biofilm} is intrinsic biofilm conductance (mS) ($G_{\text{Biofilm}} = G_{\text{Biofilm (obs)}} - G_{\text{control}}$), L_f is the biofilm thickness (μm), L is the length of the electrodes (1.5 cm), and a is half of the

non-conductive gap between two electrodes (25 μm). For the steady-state biofilm anode at each buffer condition, biofilm conductivity was measured at least in triplicate.

2.3. Biofilm thickness

Biofilm thickness (L_f) was measured with the method proposed by Bonanni et al. (2013) using the change of electrical resistance between the surface of biofilms and microelectrodes; this method is very useful to measure biofilm thickness without scarifying biofilm samples. Stainless steel needle (a 100 μm diameter) connected with a motorized micromanipulator (MM33, Unisense A/S, Denmark) was positioned very close to the surface of the biofilm anode, and then moved toward gold electrodes at a step size of 5 μm using Unisense SensorTrace Suite software (Unisense A/S, Denmark). Literature provide detailed information on L_f measurement (Dhar et al., 2016b, Lee et al., 2016). At least triplicate measurements of biofilm thickness were carried out for the state-state biofilm anode at each buffer condition.

2.4. pH gradient throughout the biofilm anode

For steady-state current density at different phosphate buffer concentrations, pH gradient within the biofilm anode was measured using a pH microelectrode (Unisense pH-100, Unisense A/S, Denmark) with a tip diameter of 100 μm connected with a 4-channel microsensor multimeter (Microsensor Multimeter for Unisense Sensors, 2x pA, 1x mV and 1x Temp channel, Unisense A/S, Denmark). The pH microelectrode was used in combination with an external Ag/AgCl reference electrode (Unisense REF-10, Unisense A/S, Denmark). After confirming steady-state current density at given phosphate buffer concentration we disassembled the $\text{MxC}_{\text{buffer}}$, placed the anode, the cathode and the reference electrode in a rectangular open-chamber filled with acetate medium amended with given phosphate buffer concentrations (2.5–100 mM); to accommodate the pH microelectrodes in the chamber without breaking their fragile tips, we excluded the anion exchange membrane from the chamber, and continuously purged the chamber with ultra-pure nitrogen (99.999%) to mitigate O_2 diffusion to the biofilm anode and H_2 transport from the cathode to the biofilm during pH measurements. The anode was poised at -0.2 V vs. SHE using the potentiostat to keep equivalent growth conditions during pH gradient measurements. A schematic of the experimental set-up used for pH gradient measurements was provided in Supplementary Material (Fig. S1). The pH microelectrode tip was positioned very close to the surface of the biofilm and then moved toward the biofilm at a step size of 5 μm using a motorized micromanipulator (Unisense MM33, Unisense A/S, Denmark). We measured pH profiles throughout the biofilm at least twice for each buffer concentration and averaged data. We observed that current density immediately reached at the steady state (0.64–2.38 A/m^2) after reassembling and operating the continuous $\text{MxC}_{\text{buffer}}$ with acetate medium (25 mM) amended with phosphate buffer (2.5–100 mM).

2.5. Confocal laser scanning microscopy of the biofilm anode

To prove metabolic activities of ARB within biofilm anodes at 2.5 mM and 100 mM phosphate buffer concentrations, we qualitatively assessed metabolic activity of ARB throughout biofilm anodes using a LIVE/DEAD cell stain with a confocal laser scanning microscopy (CLSM) (Zeiss LSM 5 Duo Vario Microscope). The CLSM images of a biofilm

anode were taken in the $\text{MxC}_{\text{buffer}}$ run with 25 mM acetate medium (2.5 mM phosphate buffer). In comparison, the CLSM images of a biofilm anode in the $\text{MxC}_{\text{PBS-100}}$ (an identical MxC operated with the acetate medium+100 mM phosphate buffer) were taken. We confirmed the performance of the $\text{MxC}_{\text{PBS-100}}$ was comparable to the $\text{MxC}_{\text{buffer}}$ run with the acetate medium having 100 mM phosphate buffer: the current density ($2.14 \pm 0.2 \text{ A/m}^2$), biofilm thickness ($107 \pm 20 \mu\text{m}$), and K_{bio} ($0.73 \pm 0.1 \text{ mS/cm}$). The biofilms sampled from the two MxC s were stained with Film Tracer™ LIVE/DEAD® biofilm viability kit (Life Technologies, ON, Canada) and washed with DI water for 30 min to remove excessive dyes. Then, the biofilms were visualized with the CLSM with a 20x and 10x objective, respectively. The CLSM images were taken with Zen Software (Zen 2009, Carl Zeiss AG, Jena Germany). The 2D images of the biofilms were reconstructed using the Bitplane Imaris Software (Bitplane USA, Concord MA), and we estimated biofilm thickness (biofilm layers for live and dead cells) with the software.

2.6. Acetate analysis

Acetate concentration was quantified using a gas chromatography (GC) (Model: Hewlett Packard HP 5890 Series II) equipped with flame ionization detector. All samples were analyzed in triplicate. The literature provides detailed information on operating conditions (Dhar et al., 2013).

2.7. Estimation of half-saturation potential

At different phosphate buffer concentrations, E_{KA} for the biofilm anode in the $\text{MxC}_{\text{buffer}}$ was estimated with cyclic voltammetry (CV) at a scan rate of 1 mV/s (Lee et al., 2009, Dhar et al., 2016a, Dhar et al., 2016b, Torres et al., 2008a). E_{anode} was ramped between -0.4 and $+0.2 \text{ V}$ at a scan rate of 1 mV/s using the potentiostat. The current and anode potential were recorded at every 5 s using EC-Lab for windows v 10.32 software in a personal computer connected to the potentiostat. During CV tests, acetate medium (25 mM) was fed to the biofilm anode with a HRT of 1.3 h to maintain substrate non-limiting conditions for the biofilm anode. To ensure the reproducibility of the CV data, each test was conducted at least in triplicate for the steady-state biofilm anode, and the average data is reported.

2.8. Simulation of the Nernst-Monod model

The current density in response to anode potential was simulated with the Nernst-Monod equation under non-substrate limiting conditions (Eq. (2)) (Lee et al., 2009, Dhar et al., 2016a, Dhar et al., 2016b, Torres et al., 2008a).

$$j = j_{\text{max}} \left[\frac{1}{1 + \exp\left(-\frac{F}{RT}(E_{\text{anode}} - E_{\text{KA}})\right)} \right] \quad (2)$$

where, j is the current density (A/m^2), j_{max} is the maximum current density (A/m^2), E_{anode} is the anode potential (V), E_{KA} is half-saturation potential of biofilm anode at $j = j_{\text{max}}/2$ (V), R is the ideal gas constant (8.3145 J/mol-K), F is the Faraday's constant ($96,485 \text{ C/mol e}^-$), T is temperature ($298.15, \text{ K}$). The Nernst-Monod equation assumes that the energy loss in EET

is negligible (Lee et al., 2009, Dhar et al., 2016b, Torres et al., 2008a), which means CV simulation with the Nernst-Monod equation is only valid for high K_{bio} biofilm anodes.

2.9. Microbial community analysis

Biofilm samples were collected from the mother MxC and the MxC_{buffer} with a sterilized spatula. For MxC_{buffer} run with acetate medium having 100 mM phosphate buffer, we sampled biofilms at steady state conditions (day 27). To ensure that the steady-state current density is not affected by the biofilm sampling, we kept operating the MxC_{buffer} with the same acetate medium (100 mM phosphate buffer) for additional 8 days before decreasing phosphate buffer concentration down to 50 mM (at day 35). Microbial community structures were analyzed with Illumina MiSeq PE250 approach by targeting 16S rRNA (Pitkänen et al., 2013). The total RNA was extracted from biofilms as previously described from the literature (Caporaso et al., 2011). Sequence reads were grouped at a 97% similarity and the consensus sequences were then identified using Mothur and the Silva database as a reference (Schloss et al., 2009, Quast et al., 2012). Rare members (less than 10 sequences) were excluded from the calculation of the relative abundance. Sequences were analyzed using Blast (<http://www.ncbi.nlm.nih.gov/BLAST>) and RDP classifier further confirm their phylogenetic affiliation and to classify sequences at a low taxonomic level (genus and species) whenever possible (Wang et al., 2007).

3. Results and discussion

3.1. Microbial community, biofilm thickness and current density

Geobacter spp. was highly enriched in biofilm anodes for a mother MxC and the MxC_{buffer} run with 100 mM phosphate buffer concentration. In the biofilm anode of the MxC_{buffer}, *Geobacter* genus was 78% of 36,823 rRNA sequencing reads (Table 1), which supports that *Geobacter* is the main ARB for the biofilm anode. Decrease of phosphate buffer might change microbial community structure on the biofilm anode, but major change of biofilm community would not occur for short period (~2 weeks) in the biofilm anode where growth medium and anode potential significantly affecting biofilm community were not changed (Dhar et al., 2016a, Dhar et al., 2016b, Torres et al., 2008a, Torres et al., 2009, Commault et al., 2013, Zhang et al., 2011). Previous studies also presented *Geobacter* genus was consistently main ARB in biofilm anodes grown at different phosphate buffer concentrations when biofilms were proliferated at fixed anode potential (−0.15 to −0.2 V vs SHE) using acetate medium.

The biofilm in the MxC_{buffer} was relatively thick at 119–134 μm during the experiments (Table 2), but biofilm thickness was not changed much to phosphate buffer concentrations. As expected, the steady-state current density in the MxC_{buffer} gradually decreased with decreasing phosphate buffer concentration (Fig. 2): $2.38 \pm 0.05 \text{ A/m}^2$ at 100 mM phosphate buffer, $1.53 \pm 0.03 \text{ A/m}^2$ at 50 mM phosphate buffer, and $0.64 \pm 0.04 \text{ A/m}^2$ at 2.5 mM phosphate buffer. Lower buffer concentration can acidify inner biofilm anodes, due to insufficient neutralization of protons accumulated from ARB's respiration in the biofilms (Torres et al., 2008b, Marcus et al., 2010, Marcus et al., 2011, Franks et al., 2009, Kim and

Lee, 2010). Acidic pH readily inhibits the activity of ARB, decreasing current density in MxCs (Franks et al., 2009, Kim and Lee, 2010, Patil et al., 2011).

3.2. pH gradient throughout the biofilm anode and CLSM images

Fig. 3 shows the pH gradients throughout the biofilm anode grown at different phosphate buffer concentrations in the MxC_{buffer} . At 100 mM phosphate buffer, no pH gradient was observed within the biofilm, indicating 100 mM phosphate buffer was enough to neutralize protons and maintain neutral pH throughout the biofilm anode. In comparison, lower phosphate buffer concentrations caused 0.3–0.5 pH differences between bulk liquid and the biofilm (Fig. 3b and c), proving local acidification in the biofilm anode at 2.5 and 50 mM phosphate buffer concentrations. It suggests that a local acidic pH of 6.5–6.7 in the inner biofilm can lead to the death of ARB and decrease current density in MxC. This result is consistent with the literature reporting current decline at acidic pHs <6.7 (Franks et al., 2009, Kim and Lee, 2010). We expected sharper pH gradient in the biofilm anode at 2.5 mM phosphate buffer than 50 mM phosphate, but there was no significant difference of pH gradient between the two phosphate conditions. Proton accumulation rate, which depends on current density and transport rates of HPO_4^{2-} and $H_2PO_4^-$, determines pH gradient in a biofilm anode (Torres et al., 2008b, Marcus et al., 2011). Higher current density establishes larger pH gradient throughout biofilm anodes at a given phosphate buffer concentration. We observed substantial decrease of current density ($0.64 A/m^2$) at 2.5 mM phosphate buffer over 50 mM phosphate. It seems that reduced generation of protons driven by the low current density would not sharpen the pH gradient at 2.5 mM phosphate buffer. This observation is consistent with the literature (Torres et al., 2008b, Babauta et al., 2011).

CLSM images in Fig. 4 qualitatively compared the microbial activities in biofilm anodes at 100 mM ($MxC_{PBS-100}$) and 2.5 mM phosphate buffer concentrations (MxC_{buffer}). The biofilm anode grown at 100 mM phosphate buffer ($MxC_{PBS-100}$) was entirely stained with green color, indicating that ARB were active throughout the biofilm (Fig. 4a). This result is consistent with the neutral pH observed throughout the biofilm anode at 100 mM phosphate buffer concentration. However, it is noted that Fig. 4(a) does not represent biofilm thickness in $MxC_{PBS-100}$ because it only covered ~5% of a 3D CLSM image (Fig. S3 in Supplementary Material). Biofilm thickness grown at 100 mM phosphate buffer ranged from 80 to 100 μm , which is consistent to an average thickness of $107 \pm 20 \mu m$ from triplicate measurements using the microelectrode method (see Table S1 in Supplementary Material). In comparison, an inner biofilm (~80 μm) mainly consisted of dead cells (red color) at 2.5 mM phosphate buffer (MxC_{buffer}) where most of the metabolically active ARB (viable cells) were located at outer layers of the biofilm (Fig. 4b). CLSM images well accords to pH gradients measured in the biofilm anode of the MxC_{buffer} , demonstrating that an inner biofilm was acidified and consequently dead cells were accumulated at the inner layer of the biofilm anode at low phosphate buffer. This result is well consistent to the literature (Sun et al., 2015) showing the presence of metabolically active ARB on the top of a biofilm anode. Accumulation of dead cells in the inner biofilm indicates that the density of metabolically active ARB significantly decreased at 2.5 mM phosphate buffer, reducing the steady-state current density in the MxC_{buffer} (Fig. 2). Decrease of phosphate buffer might change microbial community structure on the biofilm anode, but major change of biofilm

community might not occur for short period (~2 weeks) in the biofilm anode where growth medium and anode potential significantly affecting biofilm community were not changed (Dhar et al., 2016a, Dhar et al., 2016b, Torres et al., 2009, Commault et al., 2013, Zhang et al., 2011). Previous studies also presented *Geobacter* genus was main ARB in biofilm anodes grown at different phosphate buffer concentrations when biofilms were proliferated at fixed anode potential (-0.15 to -0.2 V vs SHE) using acetate medium. CLSM images well accord to pH gradients measured in the biofilm anode of the $\text{MxC}_{\text{buffer}}$, demonstrating that an inner biofilm was acidified and consequently dead cells were accumulated at the inner layer of the biofilm anode at low phosphate buffer.

3.3. Biofilm conductivity

The steady-state K_{bio} was 0.87 ± 0.03 mS/cm at 100 mM phosphate buffer in the $\text{MxC}_{\text{buffer}}$, which means that the biofilm anode was electrically conductive and EET follows Ohm's law in the biofilm anode where relatively small potential gradient (~200 mV) was built for saturated current density (Lee et al., 2016, Marcus et al., 2007). Interestingly, the steady-state K_{bio} decreased with decreasing phosphate buffer concentration (Fig. 5), and it became as small as 0.27 ± 0.03 mS/cm at 2.5 mM phosphate buffer in which an inner biofilm anode (~80 μm) became acidic and dead cells were accumulated at the inner layer of the biofilm anode (Fig. 3, Fig. 4). This result proves that biofilm conductivity is related to the biofilm density of live ARB, while more study is required to unpuzzle mechanisms coupling ARB's activity with biofilm conductivity. The literature reported that the conductivity of pili for *Geobacter sulfurreducens* increased from 37 ± 15 $\mu\text{S}/\text{cm}$ at pH 10.5 to 188 ± 34 mS/cm at pH 2 (Adhikari et al., 2016), which is opposite to K_{bio} change in our study. These different trends suggest that conductive EET would not solely depend on conductive pili in the *Geobacter*-enriched biofilm anode. Other conduction mechanisms, such as redox conduction, can be involved in conductive EET (Lee et al., 2016, Snider et al., 2012, Strycharz-Glaven and Tender, 2012, Yates et al., 2016, Phan et al., 2016). The conductivity of redox conduction depends on the redox status of extracellular cofactors (EC) (e.g., c-type cytochromes) throughout biofilm anodes. K_{bio} becomes highest when the fractions of oxidized and reduced ECs are equal in redox conduction biofilms (Yates et al., 2016, Boyd et al., 2015). pH can affect the fractions of oxidized and reduced cytochromes of *Geobacter* species (Dantas et al., 2015, Dantas et al., 2013), and protonation/deprotonation of the redox-Bohr group of cytochromes (e.g., histidine, alanine, lysine, asparagine, etc.) can also lead to a conformational change of cytochromes in *Geobacter sulfurreducens*, affecting redox potentials of cytochromes (Dantas et al., 2013, Morgado et al., 2010). We confirmed pH gradients throughout the biofilm anode at low phosphate buffer concentrations (Fig. 3), suggesting the possibility that pH gradients might build different fractions of oxidized and reduced ECs throughout the biofilm and influence K_{bio} . More study is required to explore the relationship among K_{bio} , the biofilm density of live ARB, and redox conduction (the concentration profiles of oxidized and reduced ECs) in biofilm anodes. However, our study clearly shows that K_{bio} is associated with the metabolic activity of biofilm anodes (composition of live and dead cells), implying the presence of multiple EET mechanisms (Lee et al., 2016, Steidl et al., 2016).

3.4. Half-saturation potential (E_{KA}) of the biofilm anode and CV simulations

Table 2 compared E_{KA} values for different phosphate buffer concentrations. E_{KA} pattern is well consistent to the trends of K_{bio} and current density. E_{KA} became positive at lower phosphate buffer concentrations in which K_{bio} and current density were smaller. More positive E_{KA} causes larger energy loss in biofilm anodes for saturated current density, supporting inefficient electron transfer from electron donor to the anode at the acidic biofilm anode. At 100 mM phosphate buffer ($K_{bio} = 0.87 \pm 0.03$ mS/cm), the experimental CV well matched the CV simulated with the Nernst-Monod equation, in which the energy loss in EET is assumed to be zero (Dhar et al., 2016b, Lee et al., 2016, Torres et al., 2008a), as shown in Fig. 6–(a). In comparison, the experimental CV at 2.5 mM phosphate buffer ($K_{bio} = 0.27$ mS/cm) became deviated from the simulated CV (Fig. 6–(c)), which accords to the literature suggesting more deviation between experimental and simulated CVs at smaller K_{bio} (Torres et al., 2008a). Modeling results also support K_{bio} decrease at the acidic biofilm anode created by low phosphate buffer concentration.

4. Conclusions

We studied the electrical conductivity of a *Geobacter*-enriched biofilm anode, as different proton gradients were built in the biofilm by controlling phosphate buffer concentration in a 25 mM acetate medium. The change of phosphate buffer concentration from 100 mM to 2.5 mM reduced the steady-state current density from 2.38 to 0.64 A/m². The decrease of phosphate buffer caused a pH gradient of 0.3–0.5 pH unit throughout the biofilm anode, reducing biofilm conductivity from 0.87 mS/cm to 0.27 mS/cm. CLSM images confirmed that ARB located at inner biofilms became dead at the low phosphate buffer, proving that the composition of live and dead ARB in biofilm anodes is linked to biofilm conductivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.
 (a) Schematic illustration of a dual-chamber MxC used in experiments, (b) a confocal laser-scanning microscopic image of non-conductive gap between two gold anode electrodes.

Fig 2.

The steady-state current density in a microbial electrochemical cell (MxC_{buffer}) operated in continuous mode. The black arrow indicates the day when the phosphate buffer in acetate medium was decreased from 100 mM to 50 mM. The red arrow indicates the day when the phosphate buffer in acetate medium was decreased from 50 mM to 2.5 mM.

Fig 3. pH gradients within the biofilm at different phosphate buffer concentrations. (a) 100 mM, (b) 50 mM and (c) 2.5 mM.

Fig 4. 2D Confocal laser scanning microscopy (CLSM) images of biofilm anodes at (a) 100 mM phosphate buffer (another MxC, called MxCPBS-100), (b) 2.5 mM phosphate buffer (MxCbuffer). Green color: live cells, red color: dead cells. The steady-state K_{bio} and current density were $2.14 \pm 0.2 \text{ A/m}^2$ and $0.73 \pm 0.1 \text{ mS/cm}$ for the MxCPBS-100, respectively, which were comparable to those in the MxCbuffer run with 100 mM phosphate-acetate medium. It is noted that Fig. 4(a), small part of a 3D CLSM image (Fig. S3), does not represent biofilm thickness in MxCPBS-100. Biofilm thickness was 80–100 μm estimated from the 3D CLSM image.

Fig 5.
Average current density and biofilm conductivity at different phosphate buffer concentrations.

Fig 6.

Experimental and simulated CVs using the Nernst-Monod equation (Eq. (2)) at different phosphate buffer concentration. (a) 100 mM phosphate buffer, (b) 50 mM phosphate buffer, and (c) 2.5 mM phosphate buffer

Table 1.Distribution of bacterial 16S rRNA in mother and experimental MxC_{buffer}.

Class	Genus	Mother reactor	MxC _{buffer} ^a
		<u>RNA (n = 48,183)</u>	<u>RNA (n = 36,823)</u>
Alpha-Proteobacteria	<i>Azospirillum</i>	c	1275 (3.5%)
	<i>Bradyrhizobiaceae</i> ^b	c	c
	<i>Hyphomicrobiaceae</i> ^b	c	35
	<i>Telmatospirillum</i>	c	c
Beta-Proteobacteria	<i>Alcaligenaceae</i> ^b	c	39
	<i>Comamonadaceae</i> ^b	c	28
	<i>Rhodocyclaceae</i> ^b	21	c
Delta-Proteobacteria	<i>Desulfovibrio</i>	c	158
	<i>Geobacter</i>	73185 (98%)	28807 (78%)
Gamma-Proteobacteria	<i>Aeromonas</i>	c	c
	<i>Enterobacteriaceae</i> ^b	c	c
	<i>Pseudomonas</i>	c	97
Bacteroidia	<i>Dysgonomonas</i>	c	c
	<i>Petrimonas</i>	c	c
	<i>Proteiniphilum</i>	c	c
Cloacamonae	<i>Cloacamonaceae</i> ^b	c	1074 (2.9%)
Clostridia	<i>Anaerovorax</i>	c	c
	<i>Fusibacter</i>	c	c
	<i>Ruminococcaceae</i> ^b	c	c
Flavobacteria	<i>Myroides</i>	c	c
Spirochaetes	<i>Treponema</i>	198	c

^aSample collected at 100 mM phosphate buffer.^bFamily.^c(not found or less than 20 sequences).

Table 2.

Biofilm thickness, biofilm conductivity, half-saturation potential and current density at different phosphate buffer concentration ($M \times C_{\text{buffer}}$).

Phosphate buffer concentration (mM)	Biofilm thickness, L_f (μm)	Biofilm conductivity, K_{bio} (mS/cm)	Half-saturation potential, E_{KA} (mV)
100	119 ± 26	0.87 ± 0.03	-225 ± 11
50	132 ± 34	0.61 ± 0.05	-211 ± 7
2.5	134 ± 14	0.27 ± 0.03	-191 ± 11