



The Effect of Intermittent Limiting Anodic Current Stimulation on the Electro Activity of Anodic Biofilms

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Abstract

Electro active biofilms is key components of bio electrochemical systems. Anodic biofilms formed by a potential-adaptive way (i.e., operation with constant external resistance or poised anode potentials) generally lack the ability to adapt to limiting currents. In the present study, a strategy based on intermittent limiting anodic current application was first employed to directly stimulate the current-adaptive growth of anodic biofilms, aiming to obtain electro active biofilms with high current adaptation. Series of intermittent limiting anodic current stimulations exhibit effectiveness for enhancing the electro-activity of anodic biofilms. Porous thick anodic biofilms with less extracellular polymeric substance production could be obtained by series of intermittent limiting anodic currents application from thin biofilms, generating robust anodic biofilms. The high stable power output of 4.35 W m⁻² could be achieved with robust anodic biofilms at high working current of 9.5 A m⁻². The present study show that current is a crucial factor influencing the performance of electro-active biofilms, suggesting that an alternative current-adaptive method could be developed for the growth of electro active biofilms with high performance.

Keywords: Electro active biofilms; Intermittent current stimulation; Current-adaptive growth; Microbial fuel cells; Bio-electrochemical

Introduction

Microorganisms capable of achieving extracellular electron transfer, termed exoelectrogens [1], have rapidly attracted increasing attention in bio-electrochemical systems (BESs) for converting wastes into electricity, hydrogen and other valuable chemicals [2-4]. Of particular interest has been systems related to microbial fuel cells (MFCs) [5,6], microbial electrolysis cells [7-9] and on-line microbial monitoring [10-12].

Microbial biofilms developed on the anode play a vital role for sustainable operation of BESs. Anode-attached biofilms accelerate the rate of the complex electrochemical reactions occurring at the interface between the solution and the solid electrode by “catalyzing” the oxidation of waste organic matter and thereby transporting electrons, derived from the oxidation reactions, to the cathode [13]. Unlike chemical catalysts or enzymes employed in other types of electrochemical systems, anode-attached biofilms exhibit both electrochemical catalysis and microbial metabolism. The development of anodic biofilms is directly related to the adaptive growth of microorganisms, rather than to a random stacking of cells on the anode surface. Thus, it is reasonable to believe that multiple factors, including physical, chemical, and electrochemical operating parameters [13-16], can have a significant effect on the development and electrochemical property of anode-attached biofilms. To activate MFC systems, the most commonly employed method is to, after inoculation with a bacterial source, connect a constant external resistance between the anode and the cathode, thus encouraging growth of electro active microorganisms. Alternatively, some studies employed a poised constant anode potential for the growth of electro active microorganisms [17-20], in order to activate the microbial anode and then to explore the effect of electrode potential on the development of electro active biofilms. In circuits with an external resistance or with poised anode potential, biofilm formation is the processes in which electro-active microorganisms (probably also involving non-electro active strains in systems with mixed cultures) grow adaptively in response to either time-dependent

anode potentials (e.g., in constant external resistance operations) or to constant anode potentials (e.g., in poised anode potential operations). However, electro-active biofilms developed in such a potential-adaptive way are generally fragile, lacking the ability to adapt to higher currents. For example, in MFC polarization measurements, currents are often observed to sharply decrease in high current range in some MFCs [21-23]. This lack of adaptation to higher currents in bio-anodes would enable MFCs to exhibit unusual electrochemical behaviors or to fail to function in higher current ranges.

To enhance the high current adaptability of anodic biofilms, alternative methods or approaches to biofilm growth should ideally be developed. However, when bio-anodes were subjected to a continuous limiting current, the anode potential would be rapidly polarized to more positive potentials beyond that suitable for microbial growth, enabling the current-adaptive growth failure. Some strategies based on intermittent connection/disconnection operation were recently developed to modulate the anode potential duration [24], external resistance loading [25,26] and MFC voltage [27]. Inspired by these studies, intermittent limiting anodic currents (ILACs) application was designed to stimulate the current-adaptive growth of anodic biofilms for the first time in the present study. Data showed that biofilms can endure the imposition of ILACs, and that a series of ILAC stimulations has a positive effect on the electrochemical behavior of the subsequent anodic biofilms.

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Received June 07, 2017; Accepted June 09, 2017; Published June 11, 2017

Citation: Zhang E, Yu Q, Zhang Y, Scott K, Diao G (2017) The Effect of Intermittent Limiting Anodic Current Stimulation on the Electro Activity of Anodic Biofilms. J Adv Chem Eng 7: 174. doi: [10.4172/2090-4568.1000174](https://doi.org/10.4172/2090-4568.1000174)

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Materials and Methods

MFC system

To avoid possible differences in chemical/microbial conditions for the development of anodic biofilms in individual MFCs, experiments used a multiple anode configuration in the anodic chamber of a single plexi-glass MFC. The MFC consisted of two chambers, each of volume 3 dm³ (L) (10 cm × 10 cm × 30 cm). The two electrode chambers were separated by a cationic exchange membrane (YS-1, Shenzhen) of 160 cm² area (8 cm × 20 cm). Both anode and cathode were made of carbon felt of thickness 0.25 mm (H2315, Freudenberg FCCT KG, Germany). In experiments, duplicate carbon felt anodes were separately fixed in the same anode chamber. For each anode, a saturated calomel electrode (SCE) was installed close to the anode surface (~1 cm apart) for subsequent potential measurements. If not stated otherwise, each anode area was 4 cm² (1 cm × 2 cm × 2). The cathode had a size of 5.0 cm × 10.0 cm. In such a configuration, one small anode and one large cathode constituted an MFC circuit, and all anodes shared common chemical/microbial conditions for the development of anodic biofilms (Figure S1).

MFC operation

To operate the MFC system, 2.7 L of catholyte containing 50 mM K₃Fe(CN)₆ and 100 mM KH₂PO₄ (pH adjusted to 7.0) was used in the cathode chamber, and 2.0 L anodic growth medium and 0.7 L mixed inoculum, taking from a separately operated (long-term running) MFC anode chamber, were added into the anode chamber. The long-term running MFC was initially inoculated with an anaerobic sludge (sampled from a domestic wastewater treatment plant of Tang Wang in Yangzhou city) and was operated using acetate as electron donor over six months. The growth medium was prepared using the following constituents (in grams per liter of deionized water): NaAc, 1.6; NaHCO₃, 2.5; CaCl₂·2H₂O, 0.1; KCl, 0.1; NH₄Cl, 1.5; NaH₂PO₄·H₂O, 0.6; NaCl, 0.1; MgCl₂·6H₂O, 0.1; MgSO₄·7H₂O, 0.1; MnCl₂·4H₂O, 0.005; NaMoO₄·2H₂O, 0.001; yeast extract 0.05. After startup of microbial anodes (indicated by the anode potentials), the MFC system was operated in a flow mode by continuously supplying anodic growth medium at a rate of 2 ml min⁻¹, in order to maintain chemo stat conditions in the anode chamber. For constant resistance operation, a constant resistance was connected to each anode and its corresponding cathode. For ILAC stimulations, the anode and its corresponding cathode were connected in series to a 24 V DC stabilized power supply (UTP3701, UNI-Trend Group Ltd., Guangdong), an adjustable resistor (0~100,000 Ω) and a time-cycle relay (DH48S-S, Omron). The constant ILAC magnitude (ILAC-M) was controlled by adjusting the resistor based on the calculation $ILAC-M = (V_{24V} + V_{MFC}) / R_{adj}$, where V_{24V} , V_{MFC} and R_{adj} are the voltage (24 V) of the stabilized power supply, the working voltage of the MFC and the adjustable resistance, respectively. Because the variation in V_{MFC} was less than 0.35 V (much smaller than $(V_{24V} + V_{MFC})$) during ILAC application, the variation in the ILAC-M could be ignored at a fixed adjustable resistance. The on/off time-cycle of the relay was set 1 s on and 1 s off to provide ILAC for the microbial anode. (Figure S1).

Measurements

The voltage outputs for each anode and cathode pair and the anode potential (measured relative to a saturated calomel electrode (SCE) placed in the anode chamber) were continuously measured using a multiple-channel high-impedance voltmeter (Keithley 2700), and data were recorded in every minute. Linear sweep voltammetry (LSV) for each anode was performed using a potentiostat (CHI 660)

with the SCE and the corresponding cathode as reference electrode and counter electrode, respectively. During LSV measurements, all anodes were disconnected from the circuits except for the measured anode. Electrode potentials are reported as values relative to the standard hydrogen electrode (SHE).

For scanning electronic microscopy (SEM), carbon felt anodes were removed from the chamber, and biofilms were fixed with 1% glutaraldehyde overnight, followed by dehydration with a series of graded ethanol (30, 50, 70, 80, 95, and 100%). The anode surface was observed using scanning electronic microscopy (HITACHI S-4800 SEM). Chemical oxygen demand (COD) was measured by a fast digestion spectrophotometric method with a COD digester and photometer (Lianhua 5B-3C, China). Medium pH was measured by a pH meter (PHS-3C, Leici, Shanghai).

Results and Discussion

Anode activation at different external resistance

Initially, different external resistances (10 Ω, 50 Ω, 100 Ω, 200 Ω, 300 Ω, 400 Ω, 500 Ω, 1000 Ω and 2000 Ω) were employed to activate the microbial anodes under stable chemo stat conditions with 830 ± 76 COD and 6.83 ± 0.15 pH (Figure S2). The external resistance showed a significant impact on the development of microbial anodes in terms of biofilm formation and electro activity. Lower external resistance generated higher stable currents (Figure 1C), consistent to previous studies [28,29]. Anode potential measurements showed that the increase in voltages could be mainly attributed to the development of the microbial anodes in these anode-limiting MFCs (Figure 1B). The electro catalytic behavior of the activated anodes was characterized by slow LSV (1 mV s⁻¹), after voltage stabilization for approximately 100 hours. Figure 1D shows that all activated anodes exhibited catalytic behavior for the bio-electro oxidation of acetate as shown by the appearance of an oxidation current at an onset potential of -0.25 V (vs. SHE) similar to other previous reports [21,28,30]. Significant differences in current-potential profiles were observed for anodes activated using different external resistances. Two types of electrochemical behavior can be distinguished according to current-potential profiles (Figure 1D). For anodes activated with a lower external resistance (LER-Anodes, activated with 10 Ω, 50 Ω, 100 Ω, 200 Ω and 300 Ω), currents increased monotonically with positive potential shifts, to reach limiting currents in the potential range higher than *ca.* -0.05 V. In contrast, all anodes activated using higher external resistance (HER-Anodes, activated with 400 Ω, 500 Ω, 1000 Ω and 2000 Ω), produced peak currents at potentials between -0.1 V and 0 V. However, currents measured on HER-Anodes decreased significantly in the high potential range that produced stable limiting current for the LER-Anodes. Similar behavior was always been observed in our experimental systems, except that the threshold value of external resistance that differentiates the two types of current-potential profiles changed with anodes of different area (Figure S3). Amongst all activated anodes, the highest electro catalytic current, obtained by slow LSV measurements, was produced by one of the HER-Anodes, i.e., the anode-400 (Figure 1C), similar to previous studies in which the highest electro activity was obtained with an intermediate external resistance [28].

Sensitivity of HER-anodes

The maximum current measured by slow LSV represents the highest catalytic capability that an activated anode may achieve under specific operating conditions. Although high catalytic currents were obtained on some HER-Anodes (e.g., anode-400, anode-500 and anode-1000 in Figure 1D), further experiments demonstrated that HER-Anodes did

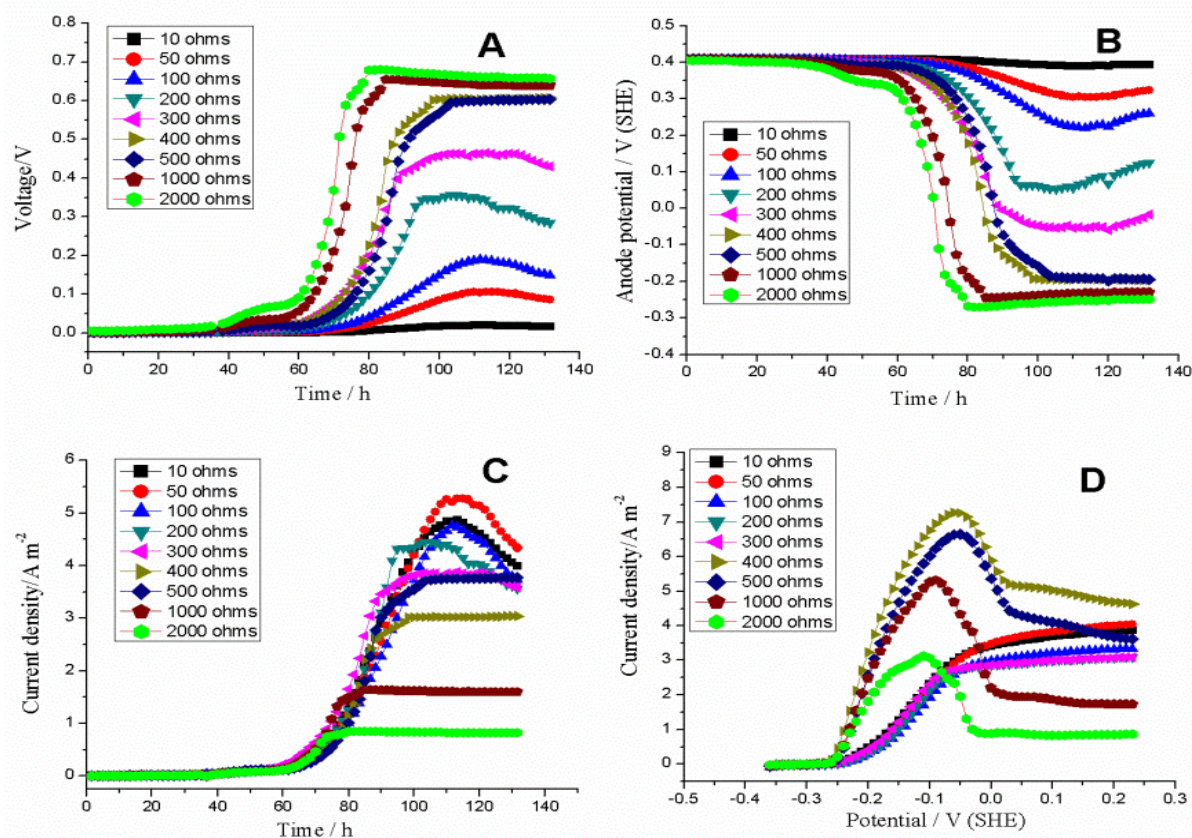


Figure 1: Variations in voltage (A), anode potential (B) and current density (C) for anodes activated with different external resistance (10 Ω , 50 Ω , 100 Ω , 200 Ω , 300 Ω , 400 Ω , 500 Ω , 1000 Ω and 2000 Ω) during startup stage; and measurements of slow (scanning rate: 1 mV s^{-1}) linear scanning voltammetry on anodes after accumulation in external resistance operation (D).

not produce stable currents at levels close to the catalytic peak currents indicated by the slow LSV measurements.

Figure 2 shows data measured on the activated anode-400 under operation with a smaller external resistance (130 Ω , enabling the anode to initially produce high currents close to peak current in insert (b) in Figure 2). The current decreased gradually from $\sim 7.8 \text{ A m}^{-2}$ to values even below the stable current generated in the activation stage with an external resistance 400 Ω (shown in insert (a) in Figure 2). The “fragile” characteristics of HER-Anodes had also been observed in the case of polarizing galvanostatically. Generally, HER-Anodes suffered from over-polarization, resulting in anode potential rapidly shift to $>1.2 \text{ V}$ (vs. SHE), within several minutes (data not shown), when subjected to a constant current as high as the peak current indicated in slow LSV curves.

Effect of a series of high ILAC stimulations

To directly stimulate the development of anode-attached biofilms by high current, ILACs were applied on LER-Anodes and HER-Anodes. To run each of ILAC stimulations, slow LSV (1 mV s^{-1}) was first performed on microbial anodes, to determine the peak current (for HER-Anodes) or the limiting current (for LER-Anodes) which were then set as the ILAC-M for the subsequent ILAC operation to stimulate the investigated anode. Each of the ILAC stimulations was performed for 20 hours. Unlike in the case in which bio-anodes were subjected to a constant limiting current, anode-attached biofilms could endure ILACs for prolonged operation without over-polarization (Figure S4). Because

the current was controlled at levels as high as the peak/limiting current of the investigated bio-anodes during ILAC application, the anode-attached biofilms were always allowed to grow under a high-current growth environment, unlike the operation by switching MFCs to operation with small external resistance in which high current generated by small external resistance decreased rapidly (Figure 2). Data showed that the series of ILAC stimulations significantly altered the electro activity of the anode-attached biofilms although differences in response to ILACs were observed between LER-Anodes and HER-Anodes. All LER-Anodes (A, B, C, D and E in Figure 3) exhibited similar evolutions of electro catalytic behavior when a series of ILAC stimulations were applied. ILAC stimulations enhanced the catalytic current of LER-Anodes from $\sim 3.0 \text{ A m}^{-2}$ to $\sim 7.5 \text{ A m}^{-2}$ at potentials of *ca.* -0.05 V (vs. SHE), but had no obvious effects in a potential range higher than 0.1 V . Significant change induced by ILAC stimulations for LER-Anodes was that the current-potential profiles exhibited peak currents at a potential of -0.05 V , similar to that of HER-Anodes. In the case of HER-Anodes, electrochemical behavior (producing peak current in current-potential profiles) did not significantly change, but the electro activity was also found to increase when subjected to ILAC stimulations, especially for anode-1000 and anode-2000 (H and I in Figure 3). Under the present stable chemo stat conditions (Figure S2), all bio-anodes with an area of 4 cm^2 ($1 \text{ cm} \times 2 \text{ cm} \times 2$), including LER-Anodes and HER-Anodes, appeared to exhibit significant increase in the electro activity when experiencing the first ILAC stimulations (Figure 3). However, further increase in the electro activity of bio-anodes was generally very limited

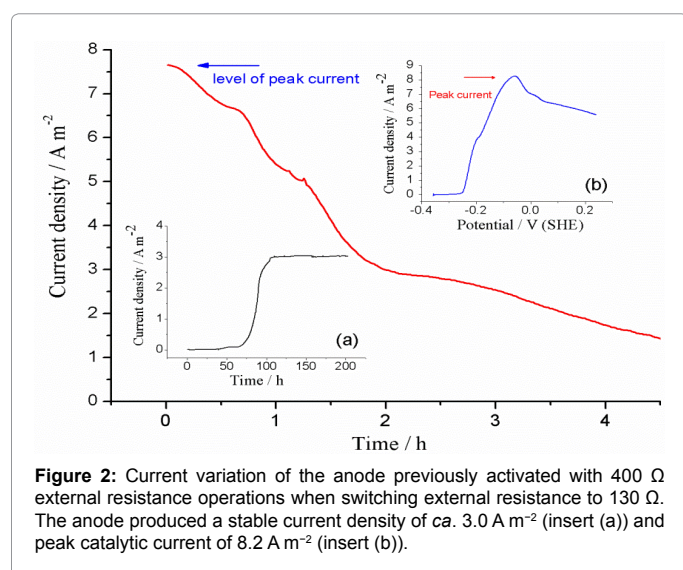


Figure 2: Current variation of the anode previously activated with 400 Ω external resistance operations when switching external resistance to 130 Ω . The anode produced a stable current density of ca. 3.0 A m^{-2} (insert (a)) and peak catalytic current of 8.2 A m^{-2} (insert (b)).

when performing high ILAC stimulations beyond the second time (Figure 3).

Figure 4 shows the SEM of anodes of 4 cm^2 that were initially activated with different external resistances (10 Ω , 50 Ω , 100 Ω , 200 Ω , 300 Ω , 400 Ω , 500 Ω , 1000 Ω and 2000 Ω) without ILAC stimulation. Craquelure-like gaps observed in SEM A, B, C, D, E, F, G and H were caused by sample drying. It can be seen that the surface of all these anodes was completely covered by a thick biofilm, except for the anode-2000 (I in Figure 4). SEM showed that the biofilms formed on anode of 4 cm^2 contained a large amount of extracellular polymeric substances, exhibiting a structure with compact feature. The compact structure with excess of extracellular polymeric substances might prevent further development of new biofilms, limiting further electro activity increase in biofilms, as shown in Figure 3.

To test the effect of ILAC stimulations on bio-anodes with more blank area for further growth of electro active biofilms, carbon felt anodes with a larger area (9 cm^2 , 3 $\text{cm} \times 1.5 \text{ cm} \times 2$) were first activated to generate a HER-Anode with a thin biofilm by operation with 500 Ω of external resistance for 4 days. When series of ILAC stimulations were applied on HER-Anodes initially with thin biofilms, the electro activity was significantly enhanced, increasing peak/limiting current from $4.8 \pm 0.4 \text{ A m}^{-2}$ to $10.5 \pm 0.5 \text{ A m}^{-2}$ (3 duplicates) (Figure 5C). During series of ILAC stimulations, thin biofilms (Figure 5A) developed to be thick biofilms, completely covering the anode surface (Figure 5B). However, compared with thick biofilms developed in external resistance operation (Figure 4), the thick biofilms (Figure 5B), developed from thin biofilms during series of ILAC stimulations, produced much less extracellular polymeric substances. Less extracellular polymeric substances production enabled the thick biofilms produced by series of ILAC stimulations to have a more porous structure (Figure 5B). In contrast, most bacterial cells in thick biofilms obtained by external resistance operation were found to be embedded in compact extracellular polymeric substances (Figures 4 and S5). Compared with compact structures in thick biofilms, porous structure in thick biofilms would be beneficial to the diffusion of nutrients and metabolic wastes [31,32], consequently reducing the effect of diffusion on high current. Remarkably, the ILAC-induced thick porous biofilms, developed from thin biofilms, showed considerable robustness. When bio-anodes with the ILAC-induced thick porous biofilms were transferred to operation with a continuous current of 9.5 A m^{-2} , close to their limiting currents,

stable power outputs of $4.35 \pm 0.27 \text{ W m}^{-2}$ in the present MFC setup with chemostat conditions (i.e., pH and concentration of organic substrate) were produced without obvious decay in performance during prolonged operation (Figure S6).

Current adaptation for robust electro-active biofilms

Bio-anodes with electro-active biofilms are key components in bio-electrochemical systems. In microbial anodes, the current is a measure of the rate of the extracellular electron transfer by electro-active microorganisms. During potential-adaptive growth of electro active biofilms, electric current, as a dependent parameter, is governed partially by the structure, diffusion, biomass and physiological activity of the electro-active biofilms [13,29,33]. For instance, current would be limited in biofilms with compact structures, slow nutrient diffusion and ineffective microorganisms. However, the present study indicated that the current could conversely influence the development of electro-active biofilms, and offer anode-attached biofilms opportunities to develop high current-adaptive capability when it was controlled as an active factor to stimulate the growth of biofilms, presumably through establishing an optimized biofilm structures, and accelerating the growth rate of microbial species with high effective electro-activity, at least in the sensitive startup period. Furthermore, to flow current was also shown to be crucial for maintaining the performance of the anodic biofilms that had evolved to have a high electro catalytic capacity, as illustrated in Figure 6. When the bio-anodes with high performance were disconnected from the circuit, no current flowed through the anode-attached biofilms. As a result, the electro activity decreased significantly (curves 2, 3 and 4 in Figure 6). Performance restoration of electro active anode-attached biofilms required current flowing through biofilms again (curve 5 in Figure 6).

In the present study, instead of potential, high currents were used to directly stimulate the development of electro-active biofilms by intermittent connection/disconnection operation. Recently, some strategies based on intermittent connection/disconnection operation were used to modulate the anode potential duration [24], external resistance loading [25,26] and MFC voltage [27]. However the current was still a dependent parameter in those reported strategies. In contrast, in the ILAC method presented here, the current functioned as an active electrochemical parameter, rather than as a dependent parameter, to directly stimulate the development of microbial anodes. ILAC stimulations were found to have positive effects on the current adaptive capacity of anodic biofilms in the present study, resulting in high electro activity and robustness. The present study suggested an alternative method for establishing anodic biofilms with high performance in future bio electrochemical system development.

Conclusions

Electro-active biofilms formed on anodes in bio electrochemical systems with different external resistance exhibited different electrochemical behavior. Microbial anodes that were activated with external resistance operation were observed to lack the ability to endure high current, but could endure the impact of ILACs without over-polarization. The application of an ILAC provided a method to directly stimulate the development of anode-attached biofilms by high currents, and has been shown to be effective for enhancing the electro-activity of anodic biofilms, especially for anodes with pre-formed thin biofilms. Robustness of anode-attached biofilms could be obtained by high current stimulations. The study showed that current, as a controlling parameter, is a crucial factor influencing the performance of electro-active biofilms, presumably through optimizing the structures in anode-attached biofilms for current production.

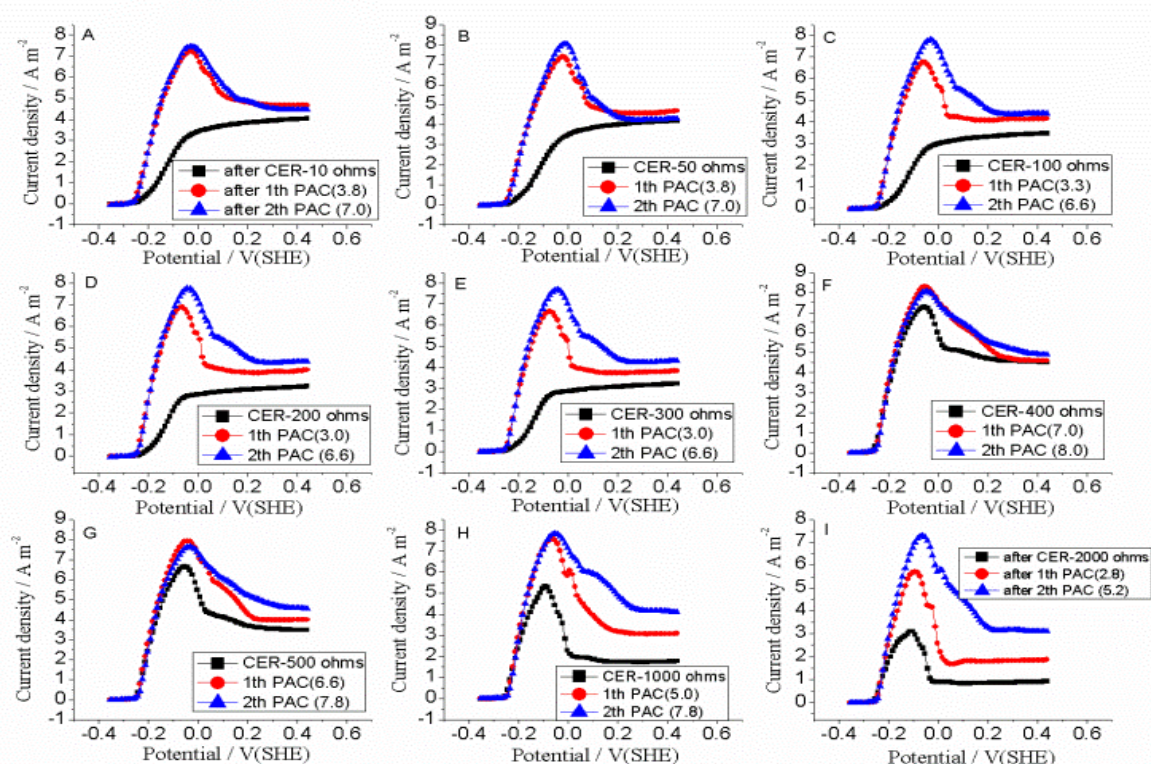


Figure 3: Evolutions of electro-activities on anodes (1 cm × 2 cm × 2) with thick biofilms activated with different external resistances (A: 10 Ω, B: 50 Ω, C: 100 Ω, D: 200 Ω, E: 300 Ω, F: 400 Ω, G: 500 Ω, H: 1000 Ω and I: 2000 Ω) when experiencing series of high ILAC stimulations. The electro-activities were characterized by slow LSV (scan rate: 1 mV s⁻¹). Taking figure A as an example to explain the legends: 'CER-10 ohms' refers to the measurement on the anode after pre-activation with constant external resistance of 10 Ω; '1th ILAC (3.8)' refers to the measurement on the same anode experiencing the first ILAC stimulation with the current 3.8 A m⁻²; '2th ILAC (7.0)' refers to the measurement on the same anode experiencing the second ILAC stimulation with the current 7.0 A m⁻².

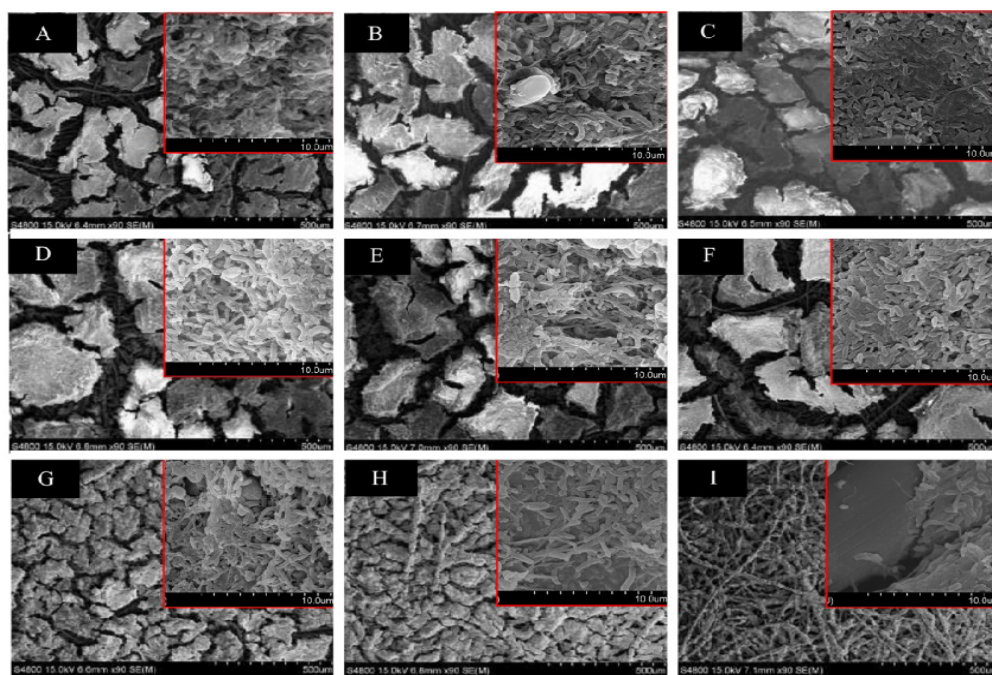
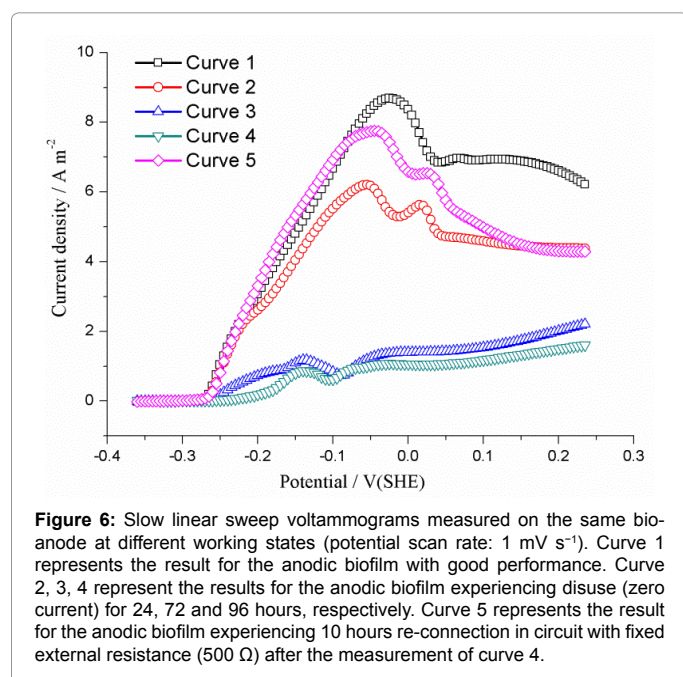
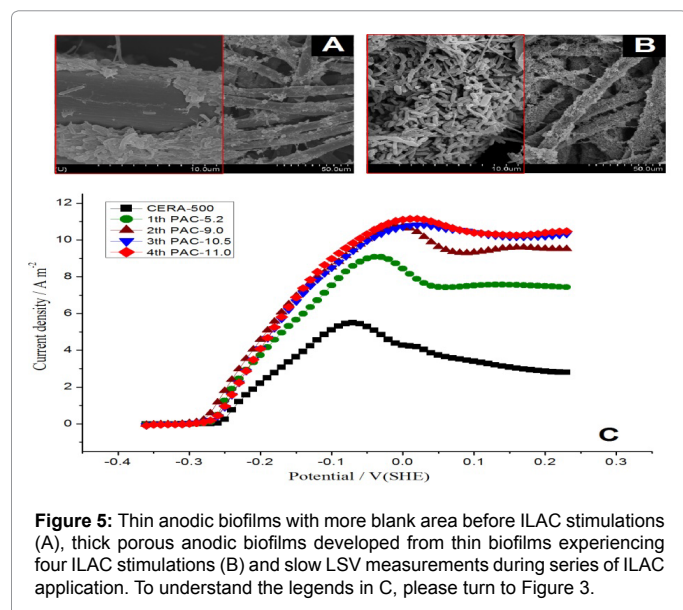


Figure 4: SEM of biofilms formed on anodes pre-activated with different external resistance (A: 10 Ω, B: 50 Ω, C: 100 Ω, D: 200 Ω, E: 300 Ω, F: 400 Ω, G: 500 Ω, H: 1000 Ω and I: 2000 Ω).



Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (Grant No. 21173184) and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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