Investigating Electrochemical Removal of Bacterial Biofilms from Stainless Steel Substrates

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ABSTRACT

Electrochemical removal of biofilms deserves attention because of its ease of use and environmentally friendly nature. We investigated the influence of electrode potential and treatment time on the removal of a 10-day old *Pseudomonas aeruginosa* biofilm formed on stainless steel 316L substrates. At electrode potentials more positive than -1.5 V vs. SCE, lower removal rates were observed and only partial removal of the biofilm was achieved during a one-minute time interval. Electrostatic repulsion between the film and electrode surface is believed to drive biofilm detachment under these conditions. However, when the biofilm-coated substrates were treated at potentials negative of -1.5 V vs. SCE, complete removal of a biofilm was achieved within seconds. Under these conditions, vigorous evolution of hydrogen gas is believed to be responsible for the film removal, mechanically detaching the bacteria and extracellular polymeric matrix from the substrate. Stainless steel substrates were also subjected to repeated cycles of biofilm formation and electrochemical removal. High removal efficiencies were maintained throughout this process suggesting the potential of the proposed technology for application on conductive surfaces in various industrial settings.

Keywords: *biofilm removal; electrochemistry; hydrogen evolution; 316L stainless steel; Pseudomonas aeruginosa*

1. INTRODUCTION

Biofilms are highly structured, three dimensional communities of microorganisms encased in extracellular polymeric substances. Bacterial biofilms form on the surfaces of equipment in food and dairy processing plants [1], marine infrastructures (*e.g.*, ships' hulls, off-shore oil platforms, fishing nets) [2], cooling pipes of thermal power plants [3], drinking-water reservoirs and water distribution systems [4], amongst many others. Biofilm formation results in blocked pipes, increased power consumption [5], corrosion of metal surfaces and reduced heat transfer through heat exchangers. Furthermore, development of biofilms in food processing environments is a potential source of contamination that may lead to spoilage and/or transmission of foodborne pathogens [6, 7].

Industries commonly tackle biofilms using mechanical treatment (*e.g.*, sponge ball cleaning, brushing and ultrasound [8]) and chemical disinfection reagents (*e.g.*, copper and organotin coatings and chlorine [2]). Conventional biofilm removal techniques have high energy demand, require frequent maintenance shutdowns, accelerate corrosion of materials and machinery and have non-reproducible performance. Chemical methods pollute the environment, endanger the health of the workers and may contaminate the final product; many biocides are not approved for sensitive processes such as those in food-associated sectors [9]. Furthermore, biocides delivered from the fluid bathing the biofilm cannot effectively penetrate and reach the full depth of the biofilm [10].

Most conventional methods apply removal forces acting on the biofilm's outer surface. However, it is believed that the weakest link in the network connecting a biofilm to a substrate is the initially adhering biomolecules and organisms [11]. Thus, a more effective cleaning method

would be to break or destabilize this weakest link [8]. In a study published in 1981, Gordon and coworkers showed that electrochemical polarization of metal surfaces affected the attachment of marine bacteria [12]. Since then, a number of investigators have demonstrated electrochemical methods as a convenient means of biofilm prevention or removal [10, 13-16]. The electrochemical methods used apply either a potentiostatic or galvanostatic mode of treatment, and are conducted in the cathodic, anodic, and block (or alternating) modes [17]. There have been earlier reports of success from applying anodic potentials to a biofilm-covered substrate [18-20]; however, it is generally accepted that cathodic and block potentials are more effective in removing a biofilm [19, 21, 22]. In addition, certain metal substrates can undergo corrosion at anodic potentials.

Electrochemical biofilm removal is generally regarded to be environmentally friendly because electrons are nontoxic reaction species [17]. However, there are numerous reports of change of pH [10, 23] and production of reactive oxygen species as a result of electrolysis [10, 22, 24]. These changes could induce corrosion of the substrate and/or contaminate the medium if the substrate needs to be polarized for long periods of time to effectively remove the biofilm [23]. Increasing the polarization potential will theoretically decrease the required treatment time for biofilm removal but will also result in higher power consumption and costs and more heat generation. Therefore, optimization of operating parameters is a necessity in the implementation of this technology.

In this work, biofilms of *P. aeruginosa* were developed on stainless steel 316L substrates. To investigate the biofilm removal kinetics, the substrates were subjected to electrochemical cathodic polarization in an aqueous solution at various potentials, for various treatment times,

and to repeated cycles of biofilm formation and removal. Microscopy techniques were used to verify the extent of biofilm removal on the treated substrates.

2. MATERIALS AND METHODS

2.1. Substrate preparation

Stainless steel 316L (SS 316L) was obtained in the form of a 1.5 cm diameter rod (Fort Wayne Metals, IN). Coin-shaped samples were cut to have equal surface area and thickness, and wet-polished successively with 120 to 1200/4000 grit sandpaper. To ensure that they were clean and free of polishing residues, the samples were sonicated in deionized (DI) water and degreased in acetone and ethanol, then dried with argon gas. The stainless steel substrates were sterilized with steam (121°C, 30 min) before they were inoculated with bacteria.

2.2. Biofilm development

The Gram negative bacterium *Pseudomonas aeruginosa* PAO1 containing a gfp (green fluorescent protein) plasmid was used to form the biofilms. A single colony from a fresh agar plate was grown overnight in Trypticase Soy Broth (TSB) at 37°C, 120 rpm. This culture was diluted 1:100 in TSB and was loaded (2 mL) into the wells of an untreated, 12-well, polystyrene flat-bottomed plate (Costar). The sterilized SS 316L substrates were subsequently placed in the wells and the plates were incubated at 37 °C for 10 days (static incubation). The media in the wells was replaced with fresh media every 24 hrs. Control SS 316L substrates were incubated in TSB without bacteria under the same conditions. All samples were prepared in triplicate.

2.3. Electrochemical measurements

All the electrochemical measurements were carried out in a single-compartment, 50 mL electrochemical cell with a graphite rod as the counter electrode (CE). A commercially available Ag/AgCl electrode (Innovative Instruments Inc.) was used as the reference electrode (RE). Phosphate buffered saline (PBS, pH 7.4, Sigma) was the electrolyte in all the experiments. The SS 316L substrate, with or without bacterial biofilm, was the working electrode (WE). Electrochemical techniques of linear voltammetry (LV), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were employed using an Ecochemie Autolab potentiostat/galvanostat/frequency response analyzer PGSTAT30/FRA2, controlled by the GPES/FRA v.4.9.5 software. EIS measurements were performed in a frequency range of 100 kHz to 10 mHz with an alternating current (AC) voltage amplitude of ± 10 mV. All electrochemical experiments were performed at 23 ±2°C and repeated at least three times.

2.4. Repeated biofilm removal

To investigate the effect of repeated electrochemical biofilm removal from the substrate, the SS 316L substrates were subjected to three cycles of biofilm formation and removal. *P. aeruginosa* biofilms were formed on the substrates for 10 days, followed by the removal of the biofilmat -3.0 V, the substrates were then subjected to two more cycles of biofilm formation and removal without further pre-treatment. The corresponding current response was recorded for each round of treatment (at -3.0 V), and the surfaces were inspected *via* fluorescence microscopy for the presence of attached bacteria.

2.5. Fluorescence microscopy

Quantification of attached bacteria to the substrate was performed *via* fluorescence microscopy. The *P. aeruginosa* strain used herein harbored a gfp plasmid (ex/em 396/508 nm) enabling direct fluorescence visualization. A Carl Zeiss Confocal Laser Scanning Microscope (CLSM 700) with a 488 nm argon laser was used to image the substrates. At least 30 images for each substrate, recorded with a 20× objective, were used for quantification of attached bacteria.

2.6. Electron microscopy

Scanning electron microscopy (SEM) was used to image the surface of the SS 316L substrate before and after biofilm removal. The biofilm-covered substrates were gently washed with PBS and fixed with 2.5% (v/v) glutaraldehyde (Fisher). The fixed samples were subsequently dehydrated with a graded series of 10 min ethanol immersions (30 to 100 v/v %) and a graded series of amyl acetate in ethanol solutions (25 to 100 v/v %). The substrates were further dried using a critical point dryer for 2 hrs and coated with 300 Å of Au-Pd coating (Hummer VI Au-Pd sputter coater). Samples were examined with a Hitachi S-4700 Field Emission-STEM (FE-STEM, Tokyo, Japan) and images were recorded using Quartz PCI v. 6.0 software.

3. RESULTS

3.1. Linear and cyclic voltammetry of clean and biofilm-coated SS 316L

The linear voltammetric (LV) profile of a freshly polished SS 316L substrate in PBS is shown in Figure 1a. The increase in cathodic current at potentials negative of -1.0 V can be related to the hydrogen evolution reaction (HER) on the SS 316L surface. With an increase in

potential to higher negative (cathodic) values, the corresponding current also increases, resulting in an increase in HER rate and thus more H_2 gas production. The HER region in Figure 1a is of interest in our study; the kinetics of potentiostatic biofilm removal was studied in this potential region, as will be explained further in the text.

Initial information on the possibility of electrochemical biofilm removal was obtained by employing cyclic voltammetry (CV). The SS 316L substrate, covered with a 10-day old biofilm, was subjected to repetitive CV polarization; the 1st and 30th sweeps are presented in Figure 1b. As illustrated in Figure 1b (dotted line), the HER current on the biofilm-covered substrate is appreciable, evidencing the presence of a porous biofilm layer that does not electrically insulate the surface of the substrate (in which case the HER current would be close to zero [25, 26]). The porous structure of this film can be seen in the scanning electron micrograph presented in Figure 1c. The 30th scan (solid line) results in a larger HER current, which is due to the increase in the free SS 316L area exposed to the electrolyte. This indicates that the biofilm was, at least partially, removed during the repetitive surface cycling.

Figure 1

3.2. Kinetics of biofilm removal at different constant voltages

The CV data in Figure 1b indicates that the biofilm removal occurs within the potential region employed. To investigate this in more detail, a series of experiments were carried out in which the removal kinetics of a 10-day old *P. aeruginosa* biofilm was investigated as a function of the applied electrode potential. In these experiments, the biofilm-covered SS 316L surface was polarized at a particular electrode potential for a specific time duration and the number of bacterial cells remaining on the substrate was quantified *via* fluorescence microscopy. The

results are presented in Figure 2. In general, the number of remaining bacteria on the substrate decreased at a higher rate at greater cathodic (removal) potentials. However, two interesting behaviors should be noted. At -0.5 V and -1.0 V polarization potentials, the number of bacteria remaining on the surface after 1 minute of treatment was significantly higher than that at -1.5 V, -2.0 V and -3.0 V. The *initial* removal kinetics was slow at potentials positive of -1.5 V (including this potential), and increased sharply for potentials negative of -1.5 V. A polarization potential of -1.5 V appears to be the inflection point; although the initial removal rate is not high at this potential, with longer treatment time (60 sec), the extent of biofilm removal reached comparable levels as those achieved at higher potentials.

Figure 2

The efficiency of biofilm removal as a function of polarization potential is also apparent from the fluorescence microscopy images; Figure 3a shows a 3D reconstruction of the biofilm on the SS 316L substrate before electrochemical treatment. The thickness of the biofilm was estimated to be 11 ± 3 μm using confocal microscopy. Representative images of substrates treated for 30 sec at -0.5 V and -2.0 V are presented in Figure 3b and 3c, respectively. The substrate treated at -0.5 V possessed a considerable amount of attached bacteria, mainly in the form of clusters, even after 1 min of treatment. When the polarization potential was increased to -2.0 V, there was little trace of attached bacteria on the surface after only 30 sec of treatment.

Figure 3

3.3. Repeated biofilm removal

The influence of repetitive biofilm formation and removal on the efficiency of the removal process was investigated. In these experiments, the substrates covered with a 10-day old *P.*

aeruginosa biofilm were polarized at -3.0 V for 60 sec to remove the biofilm layer, after which they were subjected to another round of 10-day biofilm formation (without any sample pre-treatment). This cycle was repeated three times and the current response during the biofilm removal was recorded. Curves 1, 2 and 3 in Figure 4 represent the difference between the current recorded during the biofilm removal (I) and the current response of the control sample (I_0 – curve 4). As shown, the difference between the current profiles of biofilm-coated samples and the control sample is very close to zero at each removal cycle, indicating that the substrate surface retrieves its initial condition after biofilm removal and that the electrochemical treatment does not affect the physicochemical properties of the SS 316L surface.

Figure 4

The latter statement was verified by EIS measurements (Figure 5). The spectra shown in this figure represent the response of a freshly polished substrate (open triangles), the same substrate after 10 days of biofilm formation (solid circles), and after 60 sec of biofilm removal at -3.0 V (open circles).

Figure 5

For industrial applications of the technology, it would be more technically straightforward and thus, more beneficial, to operate the electrochemical biofilm removal process at constant current (galvanostatically), rather than at constant potential (potentiostatically). To evaluate this possibility, the electrochemical biofilm removal was performed at a constant current density of -56 mA/cm^2 (or current of -0.1 A). The analysis of the sample surface subsequently confirmed that the biofilm was completely removed during electrochemical treatment, confirming that the process could also be implemented under galvanostatic conditions. The resulting variation in

electrode potential is presented in Figure 6. Upon initiation of the process, the potential rapidly increased in the cathodic direction to approximately -1.62 V, which is in the potential region of the HER (Figure 1a); this was also visually observed with the vigorous evolution of H_2 gas at the electrode surface (note that the noise on the curves is due to the formation of H_2 bubbles). Figure 6 shows that the galvanostatic profile of a SS 316L sample coated with a 10-day old biofilm is evidently different from the control substrate during the initial *ca*. 50 sec of the treatment, but approaches values close to the control with time, indicating that the biofilm has been removed (which was also visually observed). The behavior in Figure 6 is in accordance with the CV (Figure 1b) and EIS (Figure 5) measurements; namely, due to the blockage of the SS 316L surface by the biofilm, the resistance to HER is higher in comparison to the biofilm-free surface. Therefore, according to Ohm's law, the resulting potential is higher, as evidenced in Figure 6. After removing the biofilm, the resistance decreases, leading to a decrease in the resulting potential.

Figure 6

4. Discussion

In this work, we polarized a stainless steel substrate covered with a 10-day old *P. aeruginosa* biofilm and quantified the amount of attached bacteria remaining on the substrate. The results showed that polarizing the substrate at -1.0 V and -0.5 V decreased the amount of biofilm remaining on the surface but failed to completely remove the biofilm. However, by increasing the cathodic polarization potential to -1.5 V and more negative values, the efficiency of biofilm removal significantly increased. This suggests that the mechanism dominating biofilm removal at

-0.5 V and -1.0 V could be different from that at -2.0 V and -3.0 V, with -1.5 V being the inflection point.

It is believed that the main forces acting on an adhering bacterium on a polarized surface are electrostatic, electrophoretic and electroosmotic forces [8]. The electrostatic force from a negatively polarized surface (cathodic electric current) can promote detachment of bacteria if its magnitude exceeds the bacterial adhesion force [8, 21, 27]. The bacterial cells used in this study have a negative zeta potential at pH 7.4 (data not shown), thus a repulsive force can be generated between the attached bacteria and the substrate by polarizing the substrate surface so that it acquires negative charge [8, 28]. With this logic, an increase in detachment of bacteria is expected if the polarization voltage and, thus, the electrostatic repulsive forces are increased. Thus, at -0.5 V and -1.0 V, the repulsive force between the surface and the attached bacteria could be large enough to induce some detachment of the adhered biomass. Nevertheless, this force is weak and the detachment rate is slow, resulting in a large number of bacteria remaining on the surface after one minute of treatment (Figures 2 and 3).

At -1.5 V and more negative polarization potentials, another phenomenon is observed; at these potentials, the HER occurs (Figure 1a) and H_2 gas is produced on the SS 316L surface [29]. As already noted in Figure 1a, with an increase in potential towards negative values, the HER kinetics becomes faster, and thus the H_2 production rate also becomes more significant. Therefore, when the potential is increased from -1.0 V to -1.5 V, the increase in biofilm removal efficiency after 1 minute of treatment can be attributed to a shift in the mechanism dominating biofilm detachment, from electrostatic repulsion to mechanical removal by H_2 bubbles produced on the electrode surface. The biofilm formed on the SS 316L surface is very porous (Figure 1c); therefore, PBS can penetrate through the film to the SS surface, enabling the HER to occur at

potentials negative of -1.0 V (Figure 1a). MOV.1 clearly shows the hydrogen bubbles evolving from the substrate and subsequently detaching the biofilm from the surface. Although, H_2 'pumping' seems to be the predominant mechanism for biofilm removal in this potential region, it should be noted that the increase of the surface potential towards more negative values also increases the electrostatic repulsion between the bacteria and the surface, also contributing to the faster biofilm removal.

Thus, by operating in the HER region, it is possible to achieve biofilm removal in timescales on the order of seconds. Lower treatment times translate into lower power consumption and lesser changes in pH for sensitive media. Our results showed no significant change in solution pH over the 60 sec treatment period with an applied potential of -3.0 V; however, slight increase in pH (from 7.4±0.0 to 8.0±0.0) was observed when the treatment time was increased to 120 sec. It is important to note that applying this method in electrolytes with a different ionic strength and pH influences the onset of the hydrogen evolution reaction. However, once enough hydrogen bubbles are generated on the substrate, the mechanical action of the bubbles can efficiently detach the biofilm and this is not expected to change for electrolytes with different physico-chemical properties.

Furthermore, repetitive biofilm formation and removal of a 10-day old *P. aeruginosa* biofilm by polarizing the substrate in the HER region did not appear to affect the physicochemical properties of the SS 316L surface. It is noteworthy that the EIS spectra in Figure 5 (used to confirm this claim) were recorded very close to the open-circuit potential (*i.e.*, at -0.5 V) where the SS 316L surface corrodes spontaneously; therefore, the diameter of the semicircle in the resulting plot is proportional to the corresponding corrosion resistance of the surface [30]. Figure 5 shows that when the SS 316L surface was covered with the biofilm, the resulting corrosion

resistance obtained by modeling the spectra (4.44 kΩ) was significantly higher than that of the control (1.32 kΩ). This was to be expected because the biofilm partially blocked the SS 316L surface (Figure 1c), and thus decreased access of corrosive species (chloride and hydroxide anions) to the surface by increasing the diffusion resistance, resulting in a decrease in surface corrosion. After biofilm removal (Figure 5, open circles), the corrosion resistance dropped to a value of 1.37 kΩ, which is not statistically significantly different than that recorded on the freshly polished substrate (p-value > 0.9). This demonstrates not only a complete biofilm removal from the SS 316L surface using the potentiostatic treatment at -3.0 V, but also restoration of the surface to its initial, pre-biofilm state. This behavior is in accordance with the behavior recorded in our CV measurements (Figure 1b). Therefore, the electrochemical removal of the biofilm performed at high cathodic potentials (-3.0 V) does not influence the corrosion stability of the SS 316L substrate surface under the conditions employed in this experiment, *i.e.*, it does not negatively affect the properties of the protective passive oxide film formed on the SS 316L surface.

The galvanostatic experiments further demonstrated that by operating in a constant-current mode within the HER region, high biofilm removal efficiencies could be maintained. Based on a rate of 5.4 cents/kWh of electricity (in Quebec, Canada), the cost of applying the current technology (under galvanostatic conditions, -56 mA/cm^2 and for the time duration of one minute) to remove the biofilm formed on a typical plate heat exchanger with an area of 1500 m^2 was estimated to be less than 2 $CAD. Thus, using the proposed electrochemical technology for biofilm removal is economically cheap, with the advantage of avoiding harmful environmental impacts.

It is important to note that the results presented in this work are for a single species biofilm. Single species biofilm are only laboratory models and by no means represent the status of bacterial biofilms in real applications, which are typically mixed species biofilms. Furthermore, the biofilm age of 10 days used in this work may not be representative of some industrial applications. However, the same biofilm removal efficiencies were obtained with a 1-month old *P. aeruginosa* biofilm and *Staphylococcus aureus* biofilms grown for 15 days and 1 month (data not shown). Since our proposed method is based on the mechanical action of hydrogen bubbles, it is expected to work efficiently for mixed species biofilms and also for older biofilms; however, it is recommended that the threshold voltage (the voltage where HER becomes the dominating mechanism of biofilm removal) and treatment time be optimized for more complex conditions.

Substrates subjected to repetitive biofilm removal using the proposed technique were not found to be more prone to the subsequent biofilm formation (as indicated by biofilm thickness) and the biofilm subsequently formed on an already treated surface was removed with an equally high efficiency as that formed on a freshly prepared surface. It is, however, likely that initial bacterial attachment is enhanced due to the presence of EPS footprints, yet this did not affect the efficiency of biofilm removal from the substrate.

4. CONCLUSION

Biofilm formation in processing equipment is a critical issue because it decreases the production rate and hence negatively affects the profitability of an industrial plant. Depending on the type of the industrial facility and its products, a daily, weekly or monthly cleaning schedule might be required to maintain production capacity and efficiency. In this work, we presented an

electrochemistry-based method that could potentially be used to remove biofilms formed on SS 316L-made processing equipment. Specifically, we investigated the effect of applied electrode potential on the kinetics and efficiency of the biofilm removal process. It was demonstrated that by operating at potentials or current densities that promote hydrogen evolution from the biofouled surface, the biofilm can be completely removed in a matter of seconds. The removal mechanism is based predominantly on the electrochemical formation of hydrogen gas bubbles at the porous biofilm/substrate interface, which then mechanically detach the biofilm. The results showed that, under the employed experimental conditions, the substrate surface retained its physicochemical properties after biofilm removal. The presented method is very rapid and simple and requires low operating costs. Depending on the processing equipment used (heat exchangers, filters, reactors, etc.), the metal equipment body would serve as the working electrode, and a fixed or movable counter electrode can permanently be incorporated into the design.

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Figure Captions

Figure 1. (a) Linear voltammogram of a freshly polished SS 316L surface and (b) cyclic voltammograms of the biofilm-coated SS 316L electrode recorded in 0.1 M PBS (pH 7.4); 1st (dotted line) and 30th (solid line) sweep. Scan rate = 100 mV/sec. (c) SEM image of substrate coated with 10-day old *P. aeruginosa* biofilm.

Figure 2. Number of attached *P. aeruginosa* cells per cm^2 of substrate surface after electrochemical treatment at different potentials versus treatment time. Each value represents the average of 30 frames and the error bars represent 95% confidence intervals. The biofilm was removed potentiostatically at (◇) -0.5, (□) -1, (☆) -1.5, (△) -2, and (○) -3 V, in 0.1 M phosphate buffer saline (pH 7.4).

Figure 3. (a) untreated substrate (3D reconstruction) with a measured biofilm thickness of 11±3 μm, (b) substrate treated at -0.5 V for 30 sec (note the presence of bacterial clusters) and (c) substrate treated at -2.0 V for 30 sec.

Figure 4. Chronoamperometric profile of a SS 316L electrode recorded during the biofilm removal at constant potential of -3.0 V in 0.1 M PBS (pH 7.4), during first (1) second (2) and third (3) rounds of treatment. Curves 1, 2 and 3 represent the current profile of the biofilm-coated sample subtracted from the current profile of the control. Curve 4 represents the current profile of the control.

Figure 5. Electrochemical impedance spectroscopy plots of the SS 316L electrode recorded in 0.1 M PBS (pH 7.4) for (●) biofilm coated sample, (○) electrochemically treated sample (treated at -3 V for 60 sec), and (Δ) control. The spectra were recorded at -0.5 V.

Figure 6. Chronopotentiometric profile of a SS 316L electrode recorded during the biofilm removal at constant current of -0.1 A (current density = -56 mA/cm^2) in 0.1 M PBS (pH 7.4), together with the response of the control (biofilm-free) substrate.

FIGURES

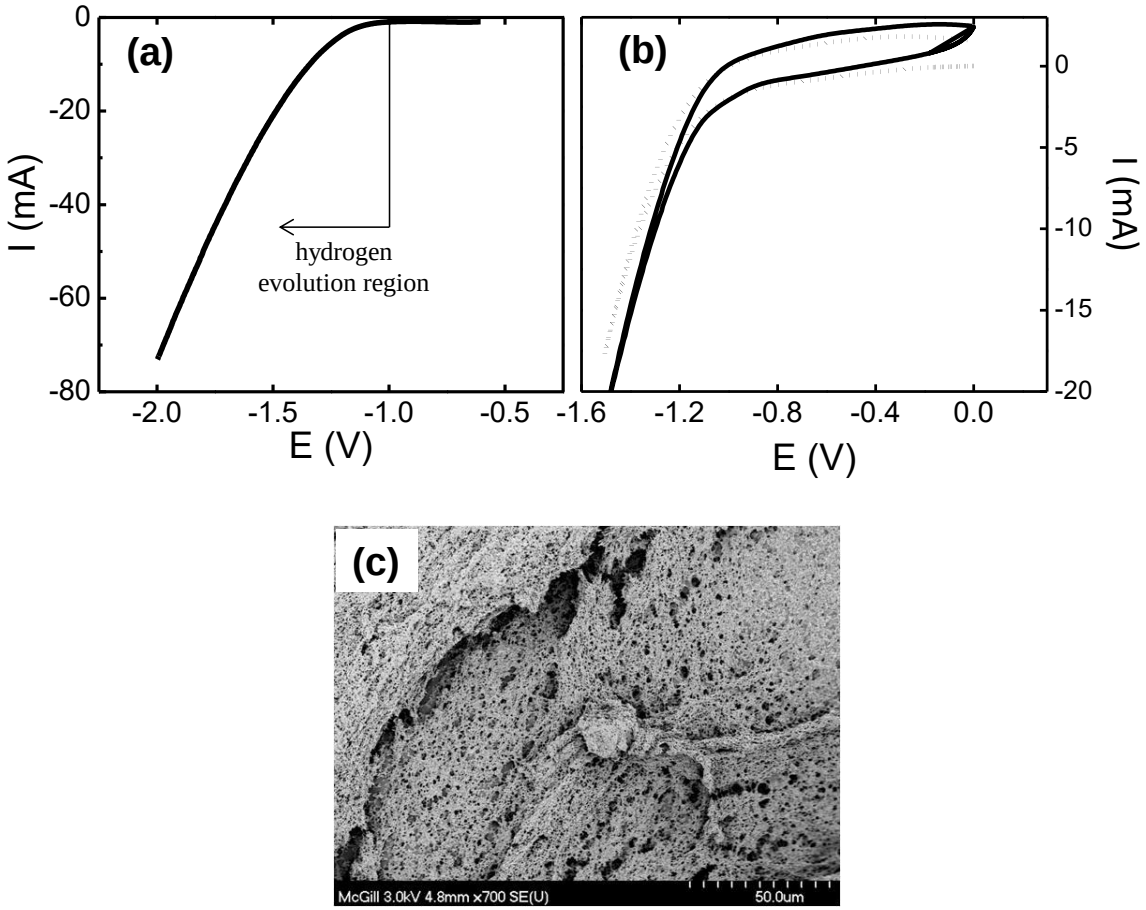

Figure 1

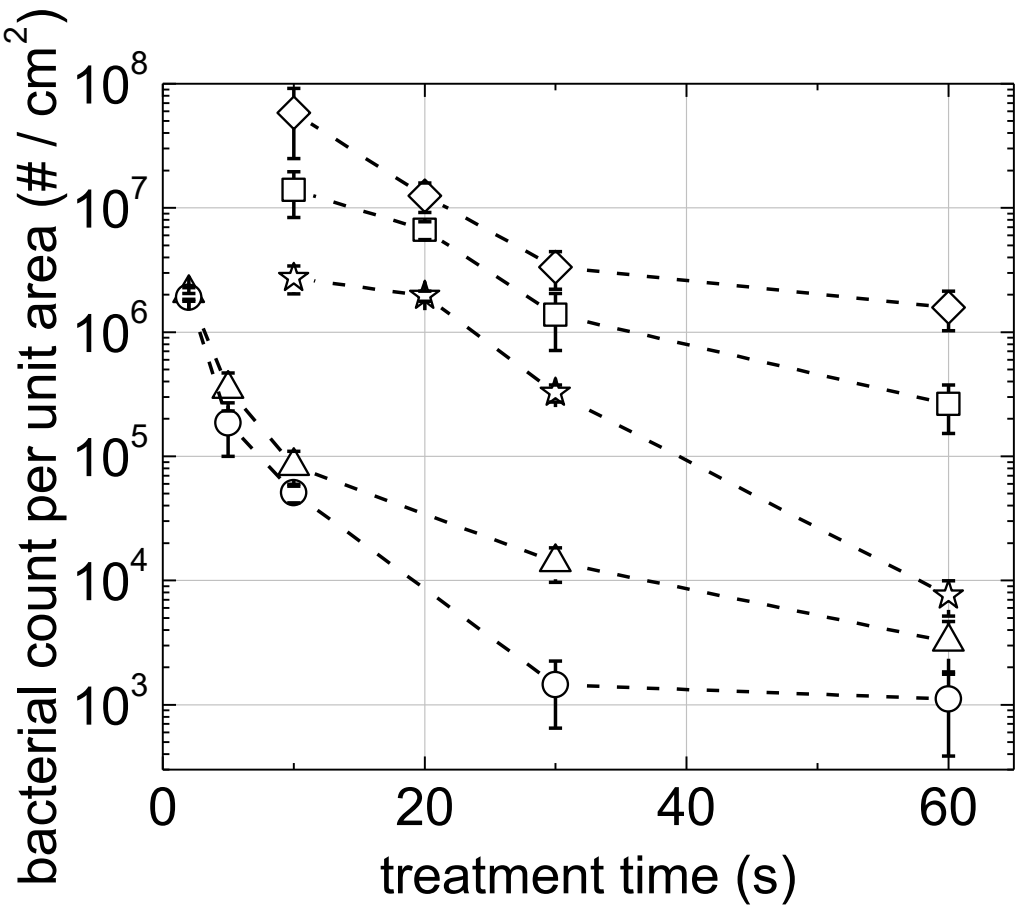
bacterial count per unit area (# / cm^2)
10^8
10^7
10^6
10^5
10^4
10^3
0
20
40
60
treatment time (s)

Figure 2

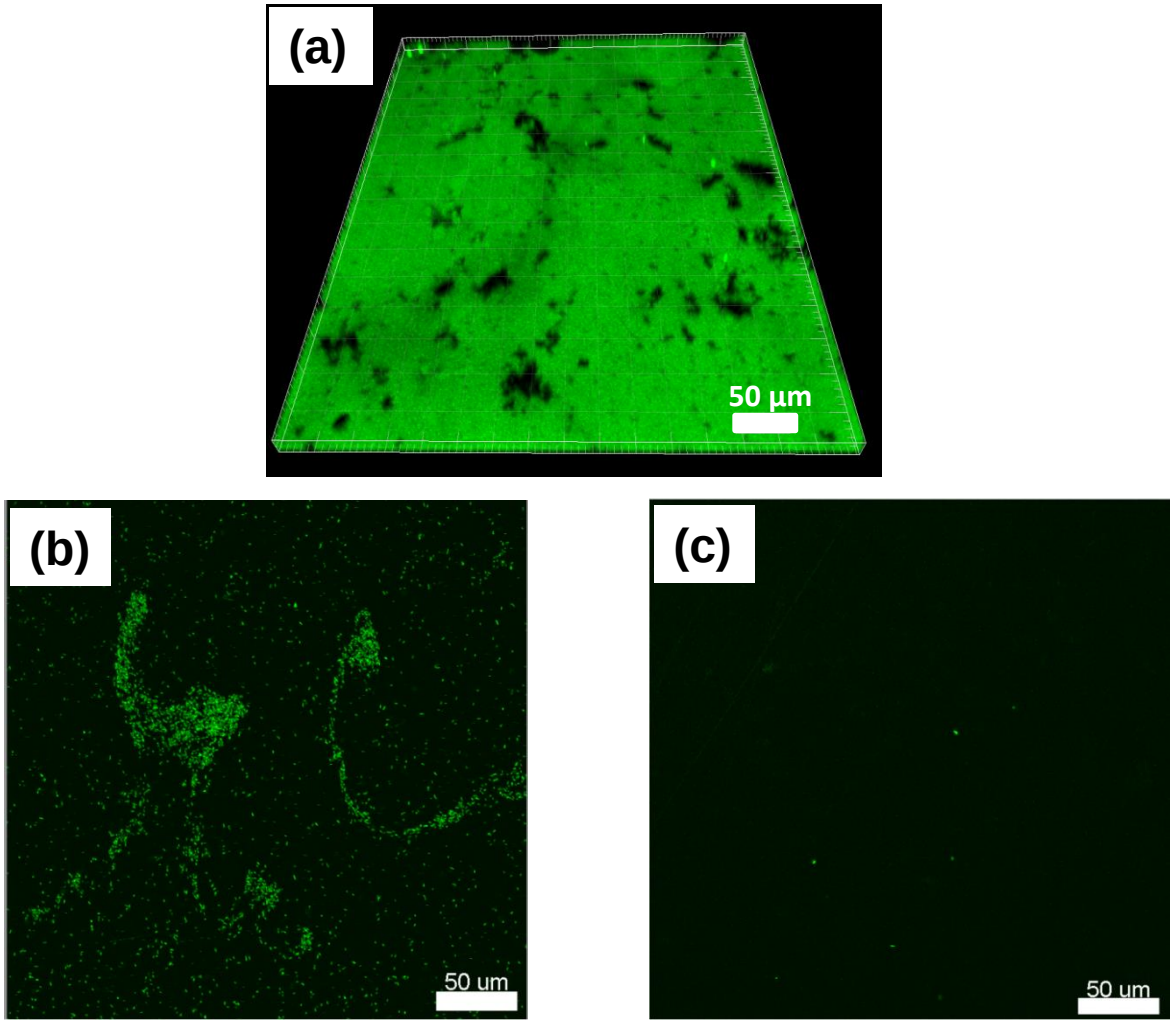

Figure 3

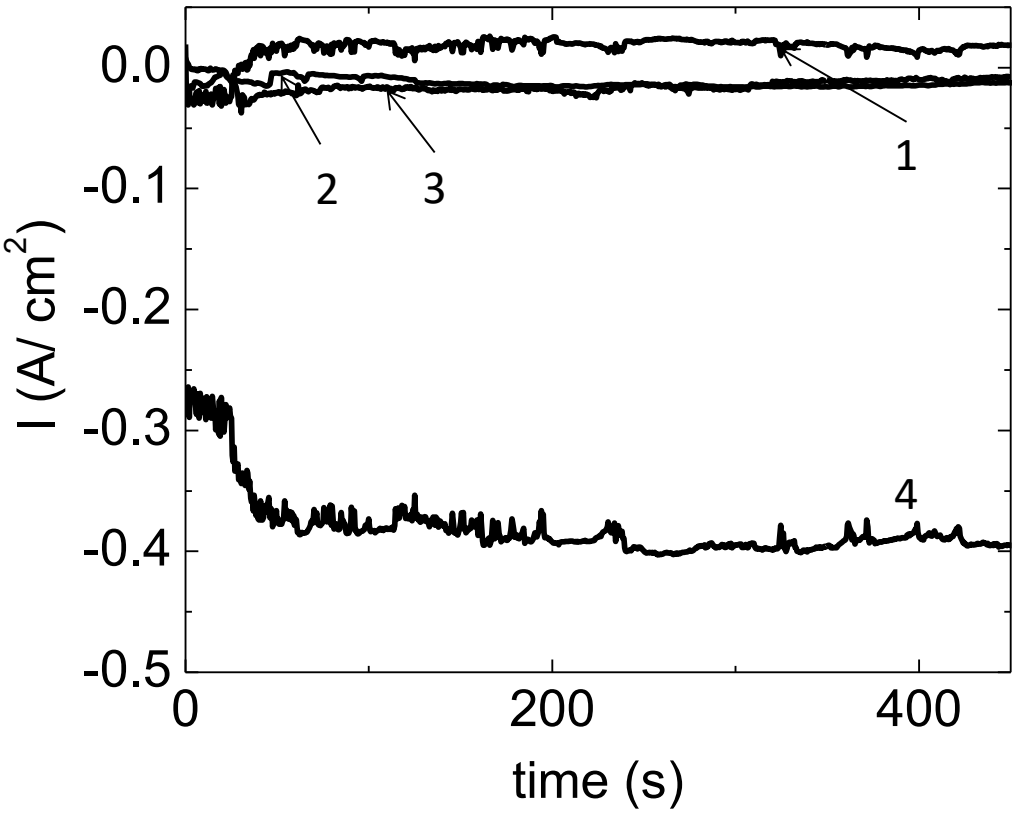

Figure 4

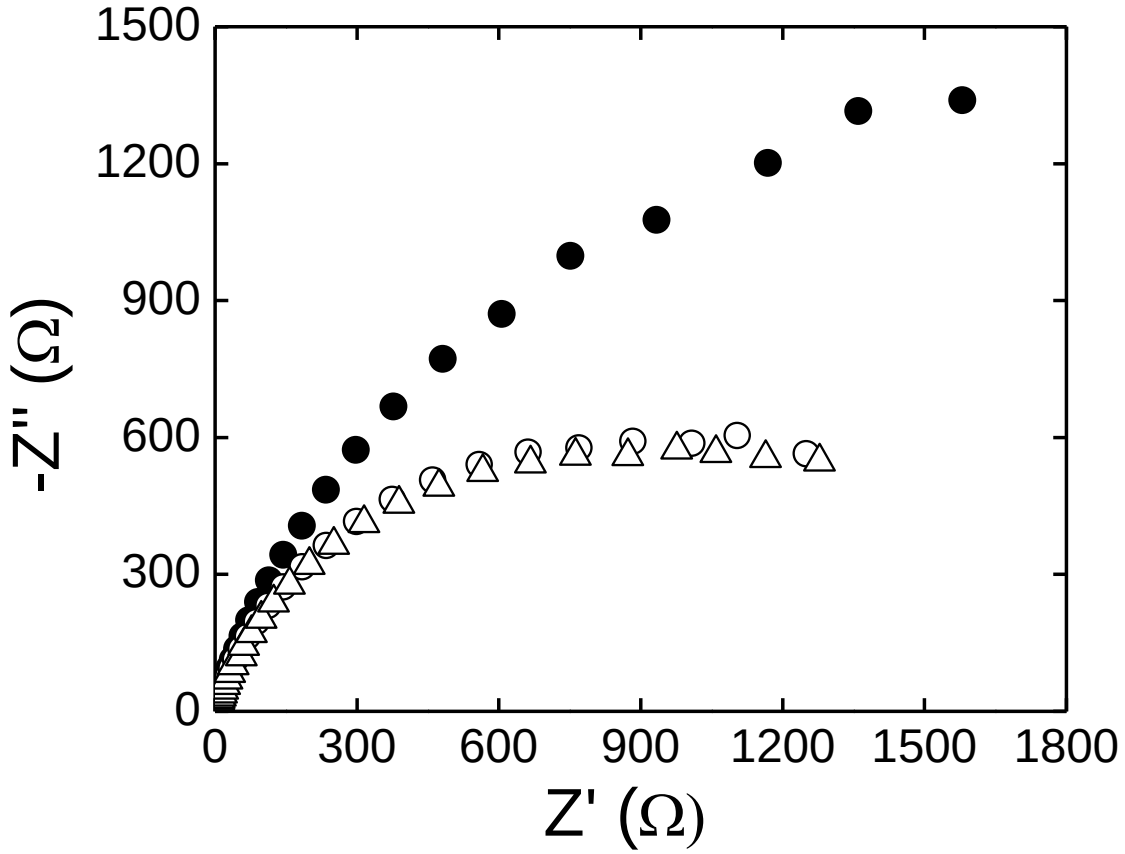

Figure 5

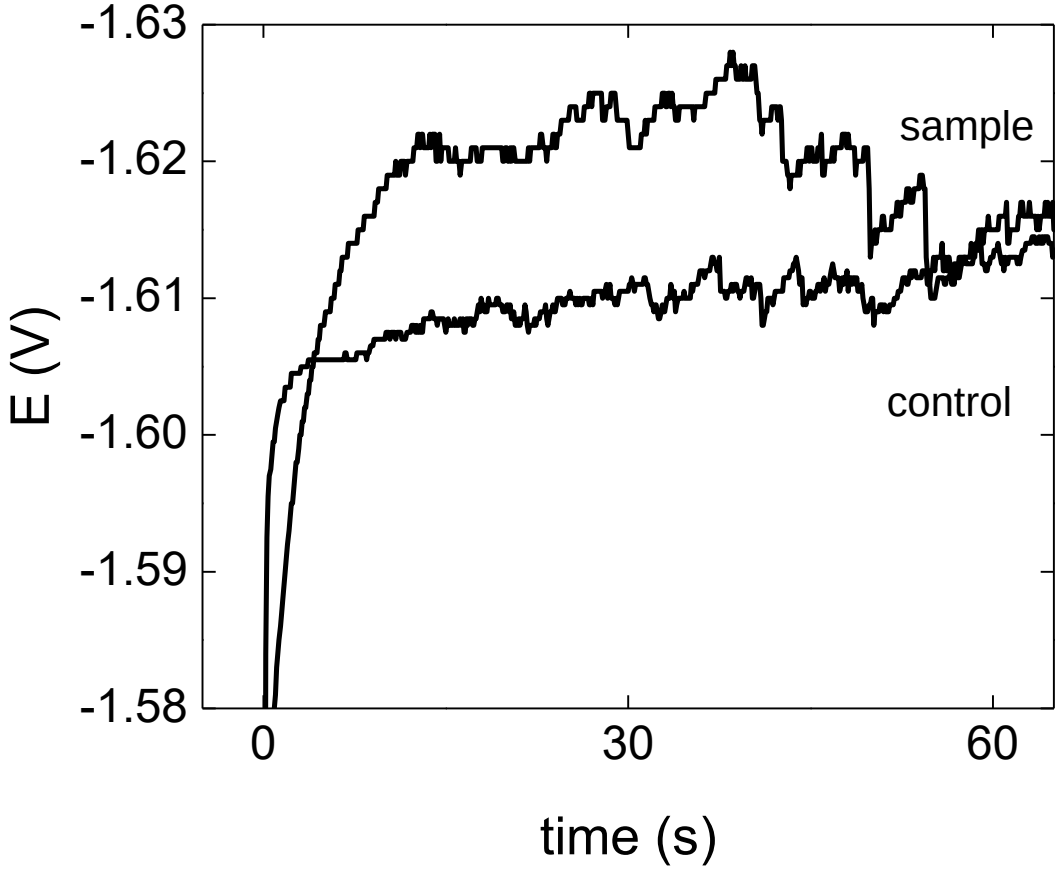

Figure 6