Technical Note: Effect of Electrochemical Impedance Spectroscopy on Microbial Biofilm Cell Numbers, Viability, and Activity☆

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INTRODUCTION

Electrochemical techniques are increasingly being used in microbiologically influenced corrosion (MIC) studies to gain a better understanding of the effects of microorganisms on the corrosion of metals. To avoid altering MIC processes by the analyses, techniques that do not damage the biofilms are preferred. Among the techniques being used to study MIC is electrochemical impedance spectroscopy (EIS). Applications of EIS in MIC studies have included monitoring corrosion over time on carbon steel exposed to bacteria,[1] carbon steel exposed to bacteria and treated with biocides,[2] stainless steel weldments exposed to bacteria,[3] and stainless steel samples exposed to natural seawater.[4]

The advantages of using EIS in MIC studies have been reviewed.[5] One of the advantages of using EIS is that small amplitude signals, within the linear response range and generally 5 mV·rms or less, are applied. Repeated EIS analysis on stainless steel samples with biofilms caused no change in the open-circuit potential after the analysis, providing indirect evidence that EIS did not damage the metal samples or the biofilms.[6] In this study, we directly tested the effect of applied sinusoidal signal on the numbers, viability, and activities of sessile bacteria on stainless steel samples. Matched-pair statistical analyses showed no significant differences between these properties of the biofilms on experimental samples (samples with sinusoidal signal applied) and control samples (samples with no signal applied).

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EXPERIMENTAL

Medium

The medium used in this study contained (per liter of deionized water) 50 mg NH_4Cl, 50 mg $MgSO_4 \cdot 7H_2O$, 5 mg $CaCl \cdot 2H_2O$, 27 mg KH_2PO_4, 15 mg MOPS buffer, 15 mg yeast extract, 15 mg peptone, 15 mg glucose, and 1 mL Hutner's mineral.[3] The pH of the medium was adjusted to 7.2 with NaOH. For analyses of colony-forming units (CFUs), 15 g/L agar was added to the medium, and the concentrations of the carbon sources used in the solid medium were increased tenfold to allow visual detection of colonies.

Bacteria

The bacteria used in this study were obtained from our laboratory culture collection of bacteria isolated from tubercles on failed steel pipes. These bacteria were analyzed by the Hewlett-Packard[(1)] microbial identification system, which compares the profile of membrane fatty acids with those contained in a computer library.[7] The bacteria selected for use in this study were; two physiologically different *Pseudomonas* spp., a *Bacillus* sp., an *Erwinia* sp., and an *Acinetobacter* sp. Bacteria were incubated for 1 to 2 days at 24°C prior to inoculation into test flasks. Two milliliters, approximately 10^9 cells, of each culture was used as the inoculum.

Experimental Flasks

Tall 1-L flasks were used as electrochemical cells. The flasks were sealed with rubber stoppers and draped with aluminum foil to prevent contamination. The rubber stopper contained ports for the salt bridge, titanium counterelectrode, and leads for the working

[(1)]Hewlett-Packard (HP) Co., Palo Alto, Ca.

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electrodes. In addition the rubber stopper had ports for medium inlet drip tubes and medium exit lines, so that the experiments could be performed under continuous flow conditions.[8] The working volume of the flasks was 500 mL, and the dilution rate was 0.5 h^{-1}. This rapid dilution rate allowed wash-out of most of the bulk-phase bacteria and formation of thick biofilms. The medium was stirred with magnetic stirrers. Flasks were sterilized with ethylene oxide gas sterilization at the University of Tennessee Medical Center.[2]

Metal Samples

The working electrodes were type 316L (UNS[3] S31603) stainless steel coupons.[4] Electrical leads were soldered to the backs of the coupons, and the coupons were embedded in epoxy. The coupons were finished with 600 grit silicone carbide paper, and the edges of the coupons were painted with lacquer to minimize crevice corrosion. The electrical leads were insulated from the solution using glass tubes. Four working electrodes were suspended in each flask.

Electrochemical Impedance Spectroscopy

Each flask contained four working electrodes. Sinusoidal signal was applied to two of the electrodes, and no signal was applied to the other two electrodes. A Solartron[5] 1250 frequency response analyzer and a Solartron 1091 electrochemical interface, controlled by a HP 300 computer, were used to generate potentials and to determine the open-circuit potential (OCP). Sinusoidal potentials of 5 mV·rms around the OCP, ranging in frequency from 5 mHz to 10 kHz, were applied to the working electrode. Five frequencies were applied per decade. After signal was applied to one of the samples, that electrode and a control electrode, where no signal was applied, were removed from the solution and microbiological analyses were performed.

Evaluation of Bacterial Populations

The procedures for determining the total numbers of bacteria by acridine orange direct counts (AODC) and the total numbers of viable bacteria by CFUs were similar to those described previously.[3] After removal of the electrodes from the flasks, O-ring sealed tubes were clamped to the coupons.[9] Basal salts solution (medium without yeast extract, glucose, or peptone added), 500 μL, was added to each tube. The solution was point sonicated 3 times in 5-s pulses at 20 percent power to remove bacteria from the surface. The suspension was serially diluted in basal salts. The diluents were filtered on 0.2-μm-pore-size Nuclepore[6] filters and stained with acridine orange to determine AODCs. The diluents were also plated on agar medium to determine the numbers of CFUs. The CFU data represent the average of duplicate plates from individual coupons.

Evaluation of Bacterial Metabolic Activity

The other two electrodes from each flask, one with the sinusoidal signal applied and the other without applied signal, were used to determine bacterial metabolic activity, as determined by lipid biosynthetic activity. The procedure for determining biosynthetic activity has been previously described.[3]

Statistical Analysis

The Student's t-test was performed on the matched-pair data. The critical t values at the 95 percent confidence level were 2.13, 2.02, and 1.94 for the 4, 5, and 6 degrees of freedom respectively, used in this study.

RESULTS AND DISCUSSION

The effects of EIS on initially colonizing biofilms and on stable biofilms were determined by performing analyses after one day of biofilm growth and after five days of growth. The experiments were designed to analyze the sessile bacteria with little contamination of bacteria from the bulk phase. Therefore a flowing system with a rapid dilution rate (0.5 h^{-1}) was used. The numbers of cells in the bulk phase, by AODC, ranged from 2×10^7 to 4×10^7 cells/mL for the one-day experiments and from 4×10^7 to 6×10^7 cells/mL for the five-day experiments. If small volumes of the bulk phase were removed with the samples, little effect would have been observed on the numbers of bacteria in the one-day biofilms, and no effect on the numbers of bacteria would have been observed in the five-day biofilms.

Stainless steel samples were used as working electrodes to avoid the build-up of corrosion products. Therefore, analyses could be performed on sessile bacteria associated with the steel surface and not with the corrosion products.

Matched-pair analysis of AODC revealed no significant difference at the 95 percent confidence level between total numbers of bacteria on electrodes where sinusoidal signal was applied and on electrodes where no signal was applied. No difference was observed for both the one-day biofilms ($n = 5$, $t = 0.60$) and for the five-day biofilms ($n = 7$, $t = 1.43$). Figure 1(a) shows the data where each matched pair (signal applied versus

[2] University of Tennessee Medical Center, Knoxville, TN.
[3] UNS numbers are listed in *Metals and Alloys in the Unified Numbering System*, published by the Society of Automotive Engineers (SAE) and cosponsored by the American Society of Testing and Materials (ASTM).
[4] Metal Samples Co., Munford Al.
[5] Solartron Instruments, Sangamo Weston Div., Irvine, CA.
[6] Nuclepore Corp., Pleasanton, CA.

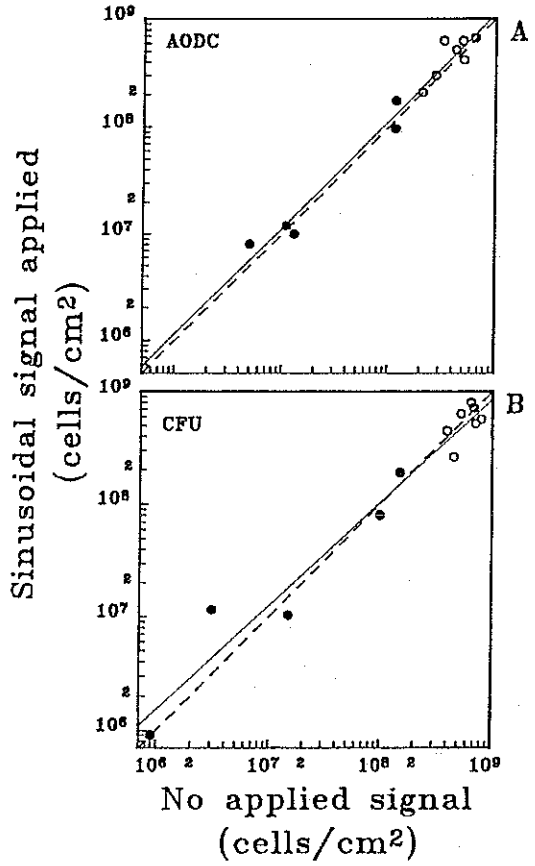

FIGURE 1. *(a) AODCs and (b) CFUs of sessile bacteria for each matched pair: bacteria from (●) one-day and (○) five-day biofilms; (——) linear regression, (– – –) the ideal 1:1 relationship.*

no signal applied) is plotted on a log–log plot. Regression analysis indicated a linear relationship ($r^2 = 0.86$) very close to the ideal 1:1 response shown as a dotted line. The results from the matched-pair analysis and the linear regression analysis demonstrated that the application of sinusoidal signal did not cause a significant decrease in the total number of cells (viable and nonviable) and therefore did not induce lysis of the sessile bacteria.

The effect of EIS on the numbers of viable bacteria in one-day biofilms and in five-day biofilms was determined by CFUs as described in the materials and methods. The matched-pair analysis revealed no significant difference at the 95 percent confidence level between the numbers of viable bacteria in the EIS-treated and the untreated samples for both the one-day biofilms ($n = 5$, $t = 0.05$) and the five-day biofilms ($n = 7$, $t = 1.55$). The data for the matched pairs are plotted in Figure 1(b). A linear relationship ($r^2 = 0.84$) with a slope close to 1 was observed for the CFUs on samples with EIS input signal applied vs samples without applied signal. These data indicated that the applied sinusoidal signal did not cause a decrease in the numbers of viable bacteria in the biofilms.

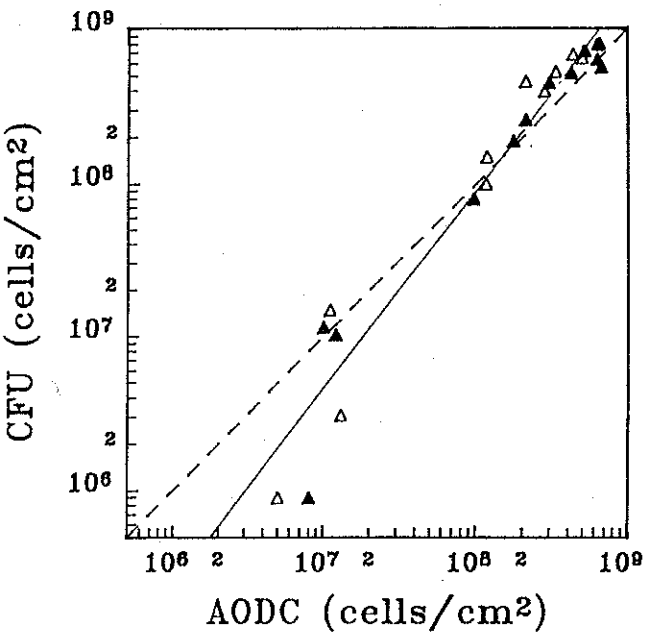

FIGURE 2. *AODCs vs CFUs: (▲) sinusoidal signal applied, (Δ) no applied signal; (——) linear regression, (– – –) the ideal 1:1 relationship.*

Figure 2 shows a linear relationship ($r^2 = 0.90$) between the AODCs and the CFUs for both the EIS-treated (closed triangles) and the untreated (open triangles) samples. These data indicate that most of the bacteria that were counted by AODC were culturable for the viable counts. This was the case for both the EIS-treated samples and the untreated samples. These data also indicated that the procedure for removing the bacteria from the surface (i.e., sonication) did not cause a reduction in the viability of the sessile bacteria. The effect of EIS on the biosynthetic activity of the sessile bacteria was determined by the incorporation of ^{14}C-acetate into total cell lipids. Matched-pair analysis revealed no significant difference at the 95 percent confidence level for the one-day biofilms ($n = 5$, $t = 1.22$), and for the five-day biofilms ($n = 6$, $t = 1.97$). Figure 3 shows the regression analysis for the activity of the EIS-treated biofilms versus the untreated biofilms ($r^2 = 0.74$). The data indicated that the application of EIS to the sample did not cause a significant change in the biosynthetic activity of the biofilms.

CONCLUSION

The results of this study indicated that EIS can be used to study mechanisms of MIC with little or no damage to the numbers of viable bacteria in a biofilm or to the activity of the bacteria.

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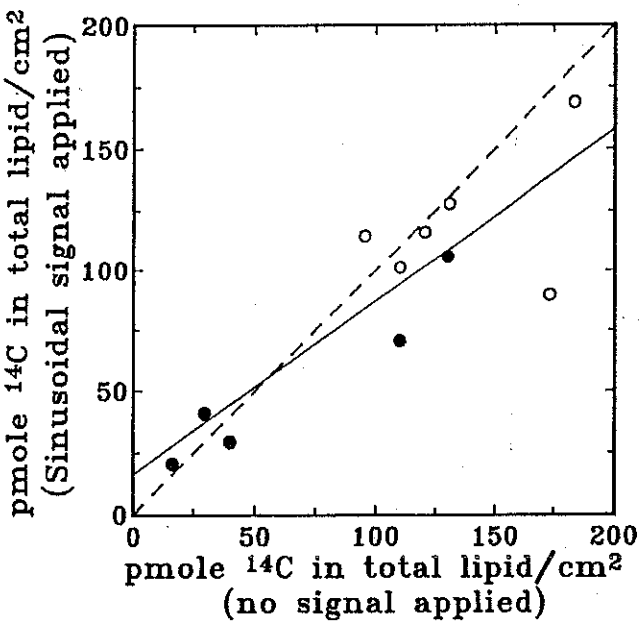

FIGURE 3. *Bacterial activity, measured by incorporation of ^{14}C-acetate into total lipids for each matched pair: bacteria from (●) one-day and (○) five-day biofilms; (——) linear regression, (– – –) the ideal 1:1 relationship.*

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