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# Eradication of *Pseudomonas aeruginosa* cells by cathodic electrochemical currents delivered with graphite electrodes

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ABSTRACT

Antibiotic resistance is a major challenge to the treatment of bacterial infections associated with medical devices and biomaterials. One important intrinsic mechanism of such resistance is the formation of persister cells that are phenotypic variants of microorganisms and highly tolerant to antibiotics. Recently, we reported a new approach to eradicating persister cells of *Pseudomonas aeruginosa* using low-level direct electrochemical current (DC) and synergy with the antibiotic tobramycin. To further understand the underlying mechanism and develop this technology toward possible medical applications, we investigated the electricidal activities of non-metallic biomaterial on persister and biofilm cells of *P. aeruginosa* using graphite-based TGON™ 805 electrodes. We employed both single and dual chamber systems to compare electrochemical factors of TGON and stainless steel 304 electrodes. The results revealed that TGON-based treatments were highly effective against *P. aeruginosa* persister cells. In the single chamber system, complete eradication of planktonic persister cells (corresponding to a 7-log killing) was achieved with 70 μA/cm$^2$ DC using TGON electrodes within 40 min of treatment, while the cell viability in biofilms was reduced by 2 logs within 1 h. The killing effects were dose and time dependent with higher current densities requiring less time. Moreover, reduction reactions were found more effective than oxidation reactions, confirming that metal cations are not indispensable, although they may facilitate cell killing. The findings of this study can help develop electrochemical technologies to eradicate persister and biofilm cells for more effective treatment of medical device and biomaterial associated infections.

**Statement of Significance**

Infections associated with medical devices and biomaterials present a major challenge due to high-level tolerance of microbes to conventional antibiotics. It is well established that such tolerance is due to the formation of dormant persister cells and multicellular structures known as biofilms. Recent studies have demonstrated electrochemical treatment as a promising alternative to eradicate bacterial infections, since the killing mechanism is independent of the growth phase of bacterial cells, but relies on various electrochemical species interplaying during the treatment. The current study investigated major bactericidal properties of the electrochemical currents mediated via TGON, a carbon-based electrode material. Up to total eradication of *Pseudomonas aeruginosa* persister cells was achieved. The new knowledge of electrochemical properties and the bioactivity of TGON may help develop new methods/devices to eradicate bacterial infections by delivering safe levels of electrochemical currents.



## 1. Introduction

Electrochemical potential difference can drive chemical fluxes across cell membranes, and deeply affect cell electrophysiology [1,2]. Previous research has demonstrated that electrochemical factors such as electric current and voltage can be delivered to

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microorganisms to eradicate pathogens from contaminated biomaterials [3]. To date, the effects of various factors including low-level direct or alternating electrochemical currents [4–6], and pulsed electric fields [7,8] have been investigated for their effects on microbes of medical relevance. For instance, low DC levels in the μA to mA range were found to kill drug-resistant bacteria [9,10] and enhance the activities of some antibiotics [11–13]. Also, electric voltages ranging from 1 to 4 V generated via inert or noble materials such as silver-nylon [14] or gold electrodes [15] were shown to be bactericidal against *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Escherichia coli*.

The electrochemical currents may fundamentally constitute a better approach for eradicating multidrug resistant cells because, in contrast to antibiotics, the currents work independently of the growth stage by physically impairing the membrane functions [16]. Recently, we reported some interesting properties of stainless steel (SS) 304, a widely used metallic biomaterial, which can generate electrochemical currents to induce oxidative stress and effectively kill the persister cells of *P. aeruginosa* PAO1. Bacterial persisters are a subpopulation of physiological variants that are highly tolerant to antibiotics [17–19]. In the phenomenon that we referred to as ECCP (electrochemical control of persister cells), DC treatment with SS304 electrodes alone reduced the viability of *P. aeruginosa* PAO1 persisters by 97.7 ± 1.7% [16], and synergistic killing activity (5-log reduction of persister viability) was achieved through concurrent treatment with DC using SS304 and 1.5 μg/mL Tob for 1 h [16]. We found that the treatment using SS304 electrodes caused structural damage to *P. aeruginosa* PAO1 cell membrane, which impaired cellular function and sensitized persister cells to antibiotics [16,20]. These findings further elucidated that the mechanisms of bacterial killing by DC treatments are associated with the electrochemical properties of the electrode materials, the generation and movement of electrochemical species (e.g. reactive oxygen species or ROS), and the synergistic interactions between released metal cations and antibiotics [21]. Although these techniques have potential for infection control, more work is needed to understand if electrochemical factors can kill persister and biofilm cells while maintaining low cytotoxicity to host tissues.

A challenge associated with metallic biomaterials such as SS304 is the possible release of electrochemical species that are biologically active and may potentially affect mammalian cells. The type of electrode material and the level of electric current/potential determine the rate of electrochemical product generation. Under our conditions, treatment with 70 μA/cm$^2$ DC using SS304 for 1 h caused the release of 0.82 μM Fe, 0.27 μM Cr, 0.10 μM Ni and 0.02 μM Mn [16]. However, the metal ions alone (without DC) were unable to kill persister cells at concentrations 15–500 times higher than those generated during DC treatments. Consistently, Harrison et al. [22,23] reported the ability of planktonic *P. aeruginosa* cells to tolerate mM levels of metal cations (including Co(II), Ni(II), Cu(II), Zn(II), Al(III), Pb(II)) for exposure times greater than 2 h. The levels of tolerance exhibited by the biofilm cells was 2–25 times higher than the minimum inhibitory concentration against normal planktonic cells, an increase that was partially attributed to the presence of persister cells in biofilms [22]. Because of the intrinsic tolerance of biofilms and persister cells to metal ions, we hypothesized that an effective strategy to eradicate recalcitrant bacterial infections may not necessarily require the use of metal cations, the accumulation and cytotoxicity of which can potentially hinder the advantage of electrochemical treatments *in vivo* [24–26]. Thus, we investigated carbon-based biomaterials as an alternative means to deliver safe electrochemical current and eradicate antibiotic-tolerant pathogens.

In this study, TGON™ 805 (henceforth, TGON), a flexible graphite sheet, was used to compare with SS304 electrodes, and to demonstrate the effectiveness of its associated electrochemical factors against persister cells and biofilms. Both single and dual chamber systems were used to differentiate the effects of low-level electrochemical currents, potentials, and byproducts (Fig. 1). We expected that a comparative study of metal and non-metal electrodes in single and dual chamber systems could provide the missing information about the necessity of metal ions for electrochemical killing of bacteria. The results showed that electrochemical currents delivered with non-metallic (carbon) electrodes have significant dose and time dependent killing effects on bacterial persister and biofilm cells; and reduction byproducts (under conditions of negative potentials) are a significant contributor to the killing effects.

## 2. Materials and methods

### 2.1. Persister isolation

The wild-type *P. aeruginosa* PAO1 (henceforth PAO1) was routinely cultured in Lysogeny Broth (LB) [27] containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. All overnight cultures were prepared in 25 mL LB and incubated at 37 °C with shaking at 200 rpm. To isolate persister cells, the overnight cultures were washed twice with 0.85% NaCl solution, and the cells were exposed to 200 μg/mL Cip for 3.5 h to kill normal cells as described previously [16]. To quantify the number of viable PAO1 persister cells, 20 μL of each sample was diluted in a 10× series using 0.85% NaCl solution, and 10 μL of each of the last 6 dilutions was plated with five replicates on LB agar plates (1.5% agar). The colony forming units (CFUs) were counted on the next day after incubation overnight at 37 °C.

### 2.2. Electrochemical properties of TGON

TGON™ 805 (Laird Technologies, IL, USA) is a flexible carbon-based material (with more than 98% carbon) with a volume resistivity as low as $11 \times 10^{-5}$ Ohm-cm. Before the material was used for the study, the electrochemical properties of TGON were investigated. First, the composition of TGON was examined using SEM (JEOL 5600, JEOL, Japan) with energy dispersive spectroscopy (EDS) (Princeton Gamma Tech, Princeton, NJ, USA). To verify that the electrochemical properties of TGON are similar to that of carbon, a polarization curve of TGON (3.5 cm × 0.95 cm; 0. 5 mm thick) was recorded over the voltage range of −2V to 1V, and compared to the reported values of graphite and SS 304 electrodes [16].

### 2.3. Effects of DC treatment using TGON

To evaluate the killing activity of DC generated with TGON electrodes, PAO1 persister cells harvested from the overnight cultures were washed twice with 0.85% NaCl solution, resuspended in 0.85% NaCl solution and treated with 35 μA/cm$^2$, 52.5 μA/cm$^2$ or 70 μA/cm$^2$ DC for 1 h as previously described [16]. All experiments were performed in triplicate. To deliver DC, a single chamber electrochemical cell (Fig. 1a) was constructed by inserting two TGON electrodes along the opposite sides of a plastic cuvette (Thermo Fisher Scientific, Pittsburg, PA, USA). A silver wire (0.015″ diameter, A-M Systems, Sequim, WA, USA) was placed in bleach for 30 min to generate an Ag/AgCl reference electrode, which was then introduced in the cuvette. DC was generated in galvanostatic mode using a WaveNow Potentiostat (Pine Research Instrumentation, Raleigh, NC, USA), where the working and counter electrodes had identical dimension and thus the same current density. The potential at the