Micron Scale Spatial Measurement of the O2 Gradient Surrounding a Bacterial Biofilm in Real Time

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ABSTRACT Bacteria alter their local chemical environment through both consumption and the production of a variety of molecules, ultimately shaping the local ecology. Molecular oxygen (O2) is a key metabolite that affects the physiology and behavior of virtually all bacteria, and its consumption often results in O2 gradients within sessile bacterial communities (biofilms). O2 plays a critical role in several bacterial phenotypes, including antibiotic tolerance; however, our understanding of O2 levels within and surrounding biofilms has been hampered by the difficulties in measuring O2 levels in real-time for extended durations and at the micron scale. Here, we developed electrochemical methodology based on scanning electrochemical microscopy to quantify the O2 gradients present above a Pseudomonas aeruginosa biofilm. These results reveal that a biofilm produces a hypoxic zone that extends hundreds of microns from the biofilm surface within minutes and that the biofilm consumes O2 at a maximum rate. Treating the biofilm with levels of the antibiotic ciprofloxacin that kill 99% of the bacteria did not affect the O2 gradient, indicating that the biofilm is highly resilient to antimicrobial treatment in regard to O2 consumption.

IMPORTANCE O2 is a fundamental environmental metabolite that affects all life on earth. While toxic to many microbes and obligately required by others, those that have appropriate physiological responses survive and can even benefit from various levels of O2, particularly in biofilm communities. Although most studies have focused on measuring O2 within biofilms, little is known about O2 gradients surrounding biofilms. Here, we developed electrochemical methodology based on scanning electrochemical microscopy to measure the O2 gradients surrounding biofilms in real time on the micron scale. Our results reveal that P. aeruginosa biofilms produce a hypoxic zone that can extend hundreds of microns from the biofilm surface and that this gradient remains even after the addition of antibiotic concentrations that eradicated 99% of viable cells. Our results provide a high resolution of the O2 gradients produced by P. aeruginosa biofilms and reveal sustained O2 consumption in the presence of antibiotics.

KEYWORDS biofilm, oxygen, antibiotics, electrochemistry, Pseudomonas aeruginosa, antibiotic resistance

Molecular oxygen (O2) is one of the most important molecules dictating bacterial lifestyle and behavior. For organisms capable of tolerating O2, it can provide a means to remove excess electrons formed during metabolism. While general fundamentals of O2 consumption are well established, the role of O2 is complex in bacterial communities, including those associated with human infection, since O2 levels vary tremendously based on the infection site and the host response (1–3). In addition,
bacteria in many infections grow as sessile communities called biofilms (4), and the three-dimensional structure of these communities can affect \( \text{O}_2 \) levels throughout the biofilm.

Previous work has shown that \( \text{O}_2 \) gradients within biofilms affect their biology (5, 6). This has prompted an examination of \( \text{O}_2 \) levels within and surrounding biofilms. In particular, stagnant biofilms rapidly deplete \( \text{O}_2 \) and waste material buildup occurs as a result of mass transport limitation at the surface of biofilms (7). Although it is clear that \( \text{O}_2 \) levels are decreased within the biofilm, the levels immediately adjacent to the biofilm surface have not been thoroughly investigated in static biofilms, in part due to the difficulties in robustly measuring \( \text{O}_2 \) with high spatial precision (5, 8–13). To address this gap in knowledge, we developed a system to spatially measure \( \text{O}_2 \) levels above a microbial biofilm in real time at the micron scale. We chose the facultative anaerobe *Pseudomonas aeruginosa* strain PA14 for these studies since this opportunistic pathogen preferentially utilizes aerobic respiration (14), and its physiology and behavior are highly influenced by \( \text{O}_2 \) availability (14, 15).

A significant challenge that was overcome is the inherent difficulty with continuously measuring \( \text{O}_2 \) over extended time periods. To address this challenge, we developed a system using electrochemical methods to measure \( \text{O}_2 \) in real-time with micron-scale spatial resolution (Fig. 1A). \( \text{O}_2 \) can be detected electrochemically through a four-electron reduction on a platinum ultramicroelectrode (UME) (Fig. 1A) (16). How-

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**FIG 1** Experimental system and SECM detection of the \( \text{O}_2 \) gradient surrounding a *P. aeruginosa* biofilm. (A) Schematic of SECM setup for measurement of \( \text{O}_2 \) gradient surrounding a *P. aeruginosa* biofilm (left), including a closeup of the SECM cell and \( \text{O}_2 \) reduction reaction at the UME tip (right). (B) The platinized UME continuously monitors bulk \( \text{O}_2 \) levels through measurement of tip current over several hours without loss of sensitivity. The \( y \) axis (ordinate) is the ratio of the tip current at each time point divided by the tip current at time zero. Each color represents biological replicates. PCM, polycarbonate membrane; DS, double-sided; UME, ultramicroelectrode.
ever, platinum UMEs readily deactivate which leads to long wait times for $O_2$ current stabilization and sub-nA current (see Fig. S1 in the supplemental material). To address this challenge, we optimized a platinization protocol that coats the UME surface with platinum particles that actively reduce $O_2$ while avoiding severe changes in the geometry of the UME surface (see Fig. S2). Importantly, our platinum UMEs had a higher electroactive area and could continuously monitor $O_2$ levels over several hours without loss of sensitivity (Fig. 1B). This is especially important because the current measured in bulk was approximated to be 205 $\mu$M; since current measured is directly proportional to $O_2$ concentration, stability ensures accurate $O_2$ measurement despite each platinized UME used only once per experiment and having slightly variable degrees of platinization or size after polishing.

We next sought to measure $O_2$ levels surrounding a *P. aeruginosa* biofilm using scanning electrochemical microscopy (SECM). The *P. aeruginosa* strain chosen for this work (*fliC*::MrT7) (17) has an inactivated flagellar motor protein, rendering the strain unable to leave the biofilm via swimming motility. Biofilms of the *P. aeruginosa* *fliC* mutant were formed on polycarbonate membranes as previously described for electrochemical studies (18). Membrane biofilms were grown for 8 h on Todd Hewitt broth (THB) agar, yielding an $\sim$3-mm-diameter nascent biofilm containing $\sim 4 \times 10^7$ bacteria (see Fig. S3). These biofilms contain fewer cells than those used in previous studies (5, 8) focused on $O_2$ consumption to better mimic biofilms observed in human infections. After formation, the membrane containing the biofilm was removed from the agar plate and attached to the bottom of a glass vial using double-sided tape and covered with $\sim 5$ ml of morpholinepropanesulfonic acid (MOPS)-glucose minimal medium.

To measure $O_2$ levels above the biofilm, a 10-$\mu$m-diameter platinized UME was approached to 40 $\mu$m above the biofilm surface using ferrocenyl methyl trimethylammonium (FcMTMA\(^{+}\); the toxicity and stability are assessed in Text S1 in the supplemental material) as the redox mediator (Fig. S4 and S5) using SECM (19). The UME tip was then poised at $\sim 0.5$ V ($O_2$ reduction potential), with a wait time of 5 min; afterward, the UME was retracted at 6 $\mu$m/s while continually measuring $O_2$ until bulk $O_2$ levels were detected, $\sim 1,400$ $\mu$m above the biofilm surface (the $O_2$ gradient calculations are detailed in Text S1). The $O_2$ levels above the biofilm resembled a sigmoidal curve with no $O_2$ detectable until $\sim 200$ $\mu$m above the biofilm (Fig. 2A; see also Fig. S6). Assuming a 10-pA minimal background current, the detection limit of the UME is $\sim 1$ $\mu$M.

For comparison, we created an $O_2$ gradient without a biofilm using a platinum electrode the same size as the biofilm as the SECM substrate. The 3-mm platinum electrode was held at three potentials (0.1, 0, and $\sim 0.5$ V versus Ag/AgCl) for 5 min, and then the $O_2$ gradient was measured as described for the biofilm (detailed in Text S1). The biofilm $O_2$ gradient was similar to the $\sim 0.5$ V poised electrode gradient, which is the potential at which $O_2$ reduction is mass transport limited at the surface of the electrode. The biofilm $O_2$ gradient was distinct from the other potentials at which $O_2$ was being consumed at a submaximal rate (i.e., limited in part by kinetics and not predominantly by mass transport). Using Comsol Multiphysics to digitally simulate $O_2$ consumption (Fig. 2B; see also Fig. S7), we approximated the flux of $O_2$ at the surface of the biofilm to be $8.2 \times 10^{-7}$ mol/cm\(^2\)/s (detailed in Text S1). Assuming each cell has a dimension of 1.5 $\mu$m $\times$ 0.8 $\mu$m, 9.8 $\times 10^{-15}$ mol/s $O_2$ or $5.9 \times 10^{10}$ molecules of $O_2$ per second were consumed by each bacterium. Collectively, these results reveal that *P. aeruginosa* biofilms produce a hypoxic zone that can extend hundreds of microns from the biofilm surface within minutes, and the biofilm consumes $O_2$ at a maximum rate.

To assess the effect of antibiotic treatment on biofilm $O_2$ consumption, we treated our biofilms with 400 times the MIC of the antibiotic ciprofloxacin (40 $\mu$g/ml) and then measured the $O_2$ gradient above the biofilm. We first confirmed that ciprofloxacin does not interfere with the electrochemical signal for $O_2$ quantification (Fig. S8; see also Text S1). Ciprofloxacin treatment of the biofilm was performed by initially adding 20 $\mu$g/ml ciprofloxacin and measuring the $O_2$ response 1.5 h after submersion in MOPS-glucose (Fig. 2C). After we observed no immediate change in signal, we treated the biofilm with another 20 $\mu$g/ml. After addition of the second dose of ciprofloxacin, the $O_2$ gradient...
FIG 2  *P. aeruginosa* rapidly produce O₂ gradients that are resilient to antibiotic treatment. (A) O₂ gradients above the surface of *P. aeruginosa* biofilms, *P. aeruginosa* biofilms treated with ciprofloxacin, and for reference a 3-mm platinum electrode poised at 0, 0.1, and −0.5 V versus Ag/AgCl (different electrode potentials correspond to various O₂ consumption rates). n = 4 biological replicates for Electrode 0.1 V, Electrode 0 V, Electrode −0.5 V, and Biofilm + Ciprofloxacin, and n = 16 biological replicates for biofilm. For all O₂ gradients, shading represents one standard deviation from the mean (solid line). (B) Digital simulation (red circles) to estimate O₂ consumption rates of the biofilm. The model was solved by Comsol Multiphysics (5.3a; COMSOL, Inc., Burlington, MA) using the electrochemical (Continued on next page)
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was measured for 50 min with no observable change in the O₂ gradient, a total of 1 h and 35 min after ciprofloxacin was first added. Despite the fact that addition of ciprofloxacin reduced the number of viable bacteria in the biofilm by 100-fold to \( \sim 2.4 \times 10^5 \) bacteria, there was no change in the O₂ gradient or O₂ consumption rates (Fig. 2A and B).

While prior work has primarily measured bulk O₂ at the biofilm/air interface, we show that, in contrast, at a stagnant biofilm/liquid interface a hypoxic region forms several hundred microns above the biofilm surface. Containing only \( \sim 4 \times 10^7 \) bacteria, our biofilms consumed O₂ at maximum rates and continued to do so despite 99% killing by ciprofloxacin. These data corroborate similar findings that bulk respiratory activity and carbon consumption persists despite antibiotic exposure (20, 21). Given this high O₂ consumption rate and the observation that biofilms of this size exist in human implant/catheter infections (22), we propose that biofilms are capable of rapidly depleting local O₂ in chronic infections even during antibiotic challenge. Ultimately, the experimental system developed in this work provides a valuable framework for studying biofilm O₂ consumption.

MATERIALS AND METHODS

**Instrumentation.** Initial electrochemistry experiments were performed using a BioLogic SECM (model M470). Biofilm experiments measuring O₂ gradients by scanning distances were done using a CHI model 920D scanning electrochemical microscope (CH Instruments). For all experiments, a three-electrode setup was used. This consisted of a 10-μm-diameter platinum UME (working electrode), Ag/AgCl (saturated KCl; reference electrode to which all potentials are referred to in all experiments), and platinum wire (counter electrode). An in-depth protocol for UME fabrication may be found elsewhere (19).

**Ultramicroelectrode fabrication and SECM cell setup.** An in-depth protocol for UME fabrication may be found elsewhere (19). Briefly, platinum (99.9% purity) wire, 10-μm diameter, temper: hard (Goodfellow Metals, Cambridge, United Kingdom; product PT005107) was used for the preparation of the SECM UME tip. The metal wire was heat sealed with a heating coil under vacuum in a glass capillary. The tip was sharpened to an RG of \( \sim 10 \), where RG is the ratio of the glass diameter to wire diameter. Prior to electrochemical experiments, UMEs were sonicated in a water bath for 30 s. Platinization significantly alters the surface and UMEs were seldom repolished and replatinized for reuse.

**Bacterial strain culture and preparation.** *P. aeruginosa* (PA14) fliC9::MrT7 mutant was obtained from a PA14 nonredundant transposon insertion mutant set (http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi) (17). Biofilms were grown in THB agar for 8 h at 37°C, at which point an \( \sim 3\)-mm-diameter biofilm formed before transfer to the SECM cell. All SECM experiments were performed using MOPS minimal media (23) containing 20 mM glucose. CFU were enumerated at the end of experimentation by removing media above the biofilm, substantially vortexing the biofilm off the polycarbonate membrane, and plating on THB agar plates overnight at 37°C.

**Platinizing UMEs.** Handmade 10 μm platinum UMEs (as described above) were sonicated in water, acetone, and water. A modified protocol for platinizing UMEs was used that may be found elsewhere (24) with an adjusted recipe for the platinization solution containing 0.250 ml of H₂PtCl₆ and 0.4 mg of Pb(NO₃)₂ up to a final volume of 7.36 ml in 1× phosphate-buffered saline (pH 7.4). Geometric and electroactive effects on the UME surface resulting from platinization were measured to confirm the stability and reproducibility of platinization. An in-depth review of platinizing electrodes can be found elsewhere (25).

**Measuring O₂.** PA14 tcn::fliC biofilms were grown as described above. After 8 h growth, the polycarbonate membrane was removed and attached to the bottom of a custom glass vial using double sided tape. UMEs were cycled in platinizing solution (same as above) from 0.2 V to \(-0.3 \) V versus Ag/AgCl at 100 mV/s until the maximum limiting current increased \( \sim 1.2 \times \) for FcMTMA⁺ oxidation (the synthesis is detailed in Text S1). After platinization and ensuring proper geometric area of the UMEs, 1 mM FcMTMA⁺ was added to MOPS-glucose media, and approximately 5 ml was added to the vial containing the biofilm. A three-electrode setup using a platinum wire counter, and Ag/AgCl reference electrodes were connected. Platinized UMEs were precisely positioned with micron-scale accuracy using SECM. SECM

**FIG 2 Legend (Continued)**

analysis module in two-dimensional axial symmetry using stationary conditions with a parametric sweep of the "d" or distance between UME tip and substrate (Fig. S5, and detailed in Text S1). (C) Changes in O₂ concentration 600 μM above a biofilm measured as a response to ciprofloxacin treatment. At 120 s, the first dose of 20 μg/ml ciprofloxacin was added (designated by red arrow). Each line represents a biological replicate. The y axis (ordinate) is the ratio of the tip current at each time point divided by current measured before ciprofloxacin addition (i.e., a value of 1 indicates no change in current after ciprofloxacin addition). There were changes immediately after ciprofloxacin addition (peaks at red arrow), likely a result of the mixing caused by addition of ciprofloxacin to the growth media above the biofilm. Importantly, the current quickly stabilized.
positions UMEs at defined distances from the biofilm surface using an electroactive mediator while observing tip current changes as a function of distance. For this work, we chose the electroactive mediator FcMTMA<sup>+</sup> since it is neither consumed by nor is toxic to <i>P. aeruginosa</i>. UMEs were poised at 0.5 V to oxidize FcMTMA<sup>+</sup>, approached within ~40 μm above the surface (within the hindered diffusion region corresponding to a decrease in signal to ~95% limiting current), and then poised at ~0.5 V and retracted at 6 μm/s to measure O<sub>2</sub> gradients. For antibiotic chronoaeropometry curves, UMEs were positioned first approximately 600 μm for the first addition of ciprofloxacin or control, approximately 1 h and 30 min elapsed after MOPS-glucose was added over the biofilm. Ciprofloxacin or control was then added quickly at approximately 2 h elapsed after MOPS-glucose was added over the biofilm. Ciprofloxacin was added slowly during this time at ~7.5 mm above the biofilm, and the stage was attached to the vial containing the biofilm was rotated 10 times in a circular motion immediately after addition. The UME was then approached approximately 300 μm above the biofilm for the second addition of ciprofloxacin, approximately 2 h elapsed after MOPS-glucose was added over the biofilm. Ciprofloxacin or control was then added quickly at ~7.5 mm above the biofilm. For both additions, a 2-min blow was given before antibiotics were added to the media and current was measured for a minimum of 1,000 s in total. O<sub>2</sub> concentration gradients were immediately measured in triplicate during each biological replicate after the second addition of antibiotics to determine the O<sub>2</sub> consumption rates.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

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