Published in final edited form as:

Anal Chem. 2017 October 17; 89(20): 11044–11052. doi:10.1021/acs.analchem.7b03050.

Real-time metabolic interactions between two bacterial species using a carbon-based pH microsensor as a scanning electrochemical microscopy (SECM) probe

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Abstract

We have developed a carbon-based, fast-response potentiometric pH microsensor for use as a scanning electrochemical microscopy (SECM) chemical probe to quantitatively map the microbial metabolic exchange between two bacterial species, commensal *Streptococcus gordonii* and pathogenic *Streptococcus mutans*. The 25- μm diameter H^+ ion-selective microelectrode or pH microprobe showed a Nernstian slope of 59 mV/pH and high selectivity against major ions such as Na^+ , K^+ , Ca^{2+} and Mg^{2+} . In addition, the unique conductive membrane composition aided us in performing an amperometric approach curve to position the probe and obtain a high-resolution pH map of the microenvironment produced by the lactate-producing *S. mutans* biofilm. The x-directional pH scan over *S. mutans* also showed the influence of the pH profile on the metabolic activity of another species, H_2O_2 -producing *S. gordonii*. When these bacterial species were placed in close spatial proximity, we observed an initial increase in the local H_2O_2 concentration of approximately $12 \pm 5 \mu\text{M}$ above *S. gordonii*, followed by a gradual decrease in H_2O_2 concentration (>30 min) to almost zero as lactate was produced, and a subsequent decrease in pH with a more pronounced metabolic output of *S. mutans*. These results were supported by gene expression and confocal fluorescence microscopic studies. Our findings illustrate that H_2O_2 -producing *S. gordonii* is dominant while the buffering capacity of saliva is valid ($\sim\text{pH } 6.0$) but is gradually taken over by *S. mutans* as the latter species slowly starts decreasing the local pH to 5.0 or less by producing lactic acid. Our observations demonstrate the unique capability of our SECM chemical probes for

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SUPPORTING INFORMATION

Schematics of the SECM setup, probe approach curve (PAC) recorded with the pH microprobe on the bacteria hydrogel biofilm in 1 mM ferrocenemethanol in artificial saliva solution (pH 7.2), calibration of pH microprobe in Britton-Robinson (BR) buffer, calibration of pH microprobe in artificial saliva before and after the SECM experiment, calibration curve of the pH microprobe in the presence of 80 μM H_2O_2 and 1 mM RuHex, pH-dependent metabolic activity of *S. gordonii* and H_2O_2 sensor calibration, fixed-distance hydrogen peroxide and pH measurement 50 μm above the dual-bacteria (Sm-Sg-Sm) biofilm, calibration curve obtained in artificial saliva with the 2 μM LysoSensorTM Yellow/Blue dextran pH probe, viability of *S. gordonii* and *S. mutans* in alginate gel, RNA isolation, cDNA synthesis, and real-time PCR., H^+ concentration vs. z-distance profile estimated from z-direction pH profile obtained on the *S. mutans* (single biofilm) and *S. gordonii* in dual biofilm.

studying real-time metabolic interactions between two bacterial species, which would not otherwise be achievable in traditional assays.

Graphical Abstract

INTRODUCTION

Mucosal surfaces in the gastrointestinal tract and oral cavities are colonized by a distinctive microbiome^{1–7} where bacterial metabolites play a critical role in interspecies interactions. For example, bacterial metabolites shape the local environment by metabolite cross feeding between specific species and therefore have an under-appreciated role in community development. Additionally, changes in the metabolic output of the community are associated with disease development such as caries and periodontal diseases. For example, *Streptococcus mutans*, a known pathogenic bacterial species in the oral microbiome, metabolizes sucrose and other carbohydrates to produce lactic acid, which lowers the surrounding pH to 5.5 or less, thus eliminating many acidintolerant bacterial species while selecting for more aciduric species, which ultimately leads to tooth decay.⁸ However, as a natural defense mechanism, a commensal bacterial species, *S. gordonii*, with the aid of pyruvate oxidase (*spxB*), metabolizes glucose and produces H_2O_2 in the presence of oxygen to keep the pathogenic *S. mutans* numbers in check.^{9–11} However, it is still unclear how the H_2O_2 -susceptible, major acid-producing cariogenic *S. mutans* survives or even thrives in the presence of H_2O_2 -producing species such as *S. gordonii*. Since the production of H_2O_2 and lactic acid are conserved among several species in the oral biofilm, they represent a functional (metabolic) output for either a health- or disease-associated polymicrobial community.^{12,13} The major challenge in understanding these local metabolic interactions is in finding the appropriate analytical tool to study real-time metabolic exchange between relevant species in a controlled spatial proximity.

In recent decades, researchers have reported^{14–16} the use of scanning electrochemical microscopy (SECM) in the study of various biological systems, including microbial biofilms.^{17–20} SECM was used in several studies^{21,22} to map the local pH on a wide variety of substrates. The general strategy in these studies was to use a thin film of mixed metal oxide as the SECM pH probe, which is easier to electrochemically deposit on a 25-µm diameter Pt or Au electrode (SECM tip). For example, Yamamoto et al reported the fabrication of a W/ WO_3 ultramicrosensor, which showed a Nernstian slope (-53.5 ± 0.5 mV/pH) with a response time of 3 s to 15 s, to study the extracellular change in pH values of endothelial cells.²¹ Wipf group has fabricated SECM pH microprobe by electrochemical deposition of hydrous iridium oxide on the exposed surface of 5 µm dia. carbon fiber and

used this probe to study change in pH near the surface of Pt and enzyme immobilized carbon microelectrode substrates under biased condition.^{23,24} Further, Ha et al used an IrO₂ SECMpH microprobe combined with an O₂ sensor (dual-tip sensor) for simultaneous imaging of oxygen and pH over the rat kidney surface.²² However, no approach curves were reported in these studies. This means that the SECM probes were positioned with the aid of an optical microscope, limiting the capabilities of SECM in positioning the probe with micrometer precision without touching the substrate. To overcome the challenge in positioning the SECM probe at a known distance above the substrate, Etienne et al developed a pH probe by filling a pulled micropipette with a pH-selective liquid membrane solution (cocktail A, pH-ionophore II Fluka) (response time ~2.5 s). They used it with shear force-based scanning electrochemical microscopy to monitor the pH during a sealing reaction at the interface between an anodized 7175 aluminum alloy and a solution containing Cr(III) and Zr(IV) species.²⁵ Later, they used a 500-nm diameter liquid membrane-based pH probe with SECM, equipped with nonoptical shear force-based distance control, to study pH changes in the microcavity by unidirectional scanning in the x, y, or z directions.²⁶ However, in all of these studies, the response time for all sensors was too slow to be effectively used as an SECM probe to obtain a high-resolution 2D pH image without compromising the spatial distortion. SICM technique was also used to monitor the pH assisted dissolution of calcite microcrystal or change in morphology by using a dual pH/electrolyte filled nanoprobe.²⁷ In another separate study, a similar electrolyte/gold coated polyaniline based SICM/SECM dual nanoprobe was used to map micropores in polyamide membrane.²⁸ Even though both these studies have reported high-resolution images, none is reported to monitor the change in the local chemical environment above live biofilm sample. In addition using SICM nanoprobe to map a highly rough biofilm surface might pose an additional challenge. Recently, our group developed a polyaniline-based dual-tip (Pt UME/pH microsensors) microprobe with SECM to quantitatively map the change in pH during the release of Ca²⁺ ions on a model substrate, bioactive glass, as well as to monitor the local change in pH of a *Sporosarcina pasteurii* biofilm-mediated calcification process.^{15,29} However, long-term stability of this electrodeposited conducting polymer remained problematic, as did the need to have two separate dedicated electrodes, one for amperometric approach curves and another for potentiometric pH measurements.

Henceforth, in the present study, we developed a solid-state, carbon-based pH microprobe as a potentiometric sensor which is also capable of performing an amperometric probe approach curve (PAC) to fix the probe-substrate distance. We used our newly developed ionophore containing carbon-based SECM pH probe, along with H₂O₂ microsensors,³⁰ to study how the microbial metabolic interactions shape species composition, eventually self-selecting for a disease-associated community. To better understand how metabolic changes in a polymicrobial consortium favor a pathogenic composition, we used a polymicrobial biofilm model comprising the commensal species *S. gordonii* and the pathogenic species *S. mutans*.

EXPERIMENTAL SECTION

Materials.

Dihydrogen hexachloroplatinate (IV) hexahydrate and 1-(2-nitrophenoxy) octane (NPOE) were purchased from Alfa Aesar. Multiwall carbon nanotubes (MWCNTs) were procured from US Research Nanomaterials, Inc. Vulcan carbon was a kind gift from Cabot Corporation. 1-Butyl-4-methylpyridinium hexafluorophosphate (ionic liquid) and polyvinyl chloride were purchased from Aldrich. Tetramethyl silane was purchased from CIL. Potassium tetrakis(4chlorophenyl)borate and dioctyl sebacate (DOS) were purchased from TCI. A proton ionophore and poly(vinyl chloride) (PVC) were purchased from Fluka and Aldrich, respectively. Alginic acid sodium salt was purchased from Sigma Aldrich. Artificial saliva (AS) (0.70 mM CaCl_2 , 0.43 mM MgCl_2 , 4 mM NaH_2PO_4 , 20 mM HEPES, 30 mM KCl) solution was freshly prepared in deionized water (18 M Ω) and stored at room temperature. Brain heart infusion (BHI) medium was purchased from BD.

Instrumentation.

All electrochemical measurements were performed by using a CHI bipotentiostat and SECM (Model # 920D, CHI, Austin, TX, USA). A newly developed pH microprobe, a 0.5-mm Pt wire were used as the working and counter electrodes respectively, along with an Ag/AgCl (1 M KCl) reference electrodes, respectively (Figure 1). Potentiometric experiments were performed with a separate high-impedance unit (EA Instruments) interfaced with SECM.

Fabrication of solid-state H^+ ion-selective microelectrode (H^+ -ISME) as an SECM pH microprobe.

A borosilicate glass capillary (o.d. 1.5 mm, i.d. 0.86 mm) was first pulled with a pipette puller (Sutter Instruments, Novato, CA, USA) and then polished to obtain an inner tip diameter of 25–30 μm ($\text{RG} < 5$) to make an H^+ ion-selective SECM microprobe. The ion-selective cocktail was made by mixing 5% proton ionophore-I, 2% potassium tetrakis(4-chlorophenyl)borate, 30% NPOE, 3% PVC, 60% Vulcan carbon powder and 500 μL tetrahydrofuran (THF). The composition was mixed thoroughly with a glass rod on a watch glass until all THF evaporated. An extra 40% of NPOE was further added to the composition to maintain consistency in a sensor paste. We then backfilled the pulled capillary with the sensor paste and pushed it to the pulled end with a Cu wire. To make electrical contact between the sensor paste and the inserted Cu wire, we added 5% Vulcan carbon in a DOS mixture to the pipet from the back opening side.²⁹ The Cu wire connection was secured by applying 10 min epoxy to the junction of the capillary end and the Cu wire. The sensor tip (Figure 1A) was polished with lens cleaning paper and cured overnight in AS (pH 7.2).

Potentiometric and amperometric characterization of H^+ -ISME (SECM pH probe).

The potentiometric performance of the pH sensor was tested by calibrating it in universal buffer from pH 10.0 to pH 4.0, Britton-Robinson (BR) buffer from pH 12.0 to pH 4.0 and AS solution from pH 7.2 to pH 4.0 by the standard addition method (with addition of 1 M lactic acid) at 23 °C. To study the effect of redox mediator (hexamine ruthenium chloride, or RuHex) and O_2 on the potentiometric performance of the sensor, we calibrated sensors in

AS in the presence of 1 mM RuHex as well as in N₂-saturated AS solution (Figure 1C). The selectivity coefficient for the sensor was determined by the fixed interference method (Table 1). Amperometric characterization of the pH probe was performed by calibrating it in 0.1 M KCl solution with the addition of RuHex by the square wave voltammetry (SWV) and constant potential method (i-t measurement). A cyclic voltammogram was also recorded in 1 mM RuHex and 0.1 M KCl to estimate the size of the H⁺-ISME (Figure 2A).

Fabrication of H₂O₂ microsensor.

A H₂O₂ sensor was prepared by using a similar procedure to that reported in our earlier study³⁰. In short, a dual Pt UME (each Pt disc having a 25-μm diameter) with RG 10 (ratio of the insulating glass diameter to the conducting Pt diameter) was fabricated. To make the H₂O₂ sensor, one of the two Pt electrodes was etched in 60:36:4 v/v saturated CaCl₂:H₂O:HCl solution to make a 10-μm cavity by applying 5 V across a graphite rod³⁰. The cavity was then cleaned in a water/ethanol mixture by sonication and subsequently dried under nitrogen. The cavity was later packed with the following paste: 10:90 w/w% of Pt-modified functionalized MWCNTs and 1-butyl-4-methylpyridinium hexafluorophosphate (ionic liquid). The dual SECM H₂O₂ probe (Figure S3A) was then calibrated in AS solution at 37 °C by applying +0.5 V vs. Ag/AgCl (Figure S3B).

Bacterial strains and growth conditions.

The streptococcal species *S. gordonii* (DL1) and *S. mutans* (UA159) were cultured on BHI agar plates at 37 °C in 5% CO₂ environments.^{1,31} Prior to all biofilm mapping experiments, *S. gordonii* and *S. mutans* were grown for 12 h in BHI and BHI with 30 mM sucrose liquid media, respectively.

Bacteria–alginate gel biofilm preparation.

For the single- and dual-species bacteria-alginate gel biofilm preparation, we collected the relevant bacterial species in their logarithmic growth phase from liquid culture and centrifuged them at 7000 rpm for 12 min at 4°C. Bacteria were washed with 10 mM phosphate-buffered saline (PBS) solution (pH 7.2) and resuspended in 10 mM PBS with 2 mM CaCl₂ (pH 7.2, 4 °C). Later, 1 mL of PBS-bacteria solution was added and vortexed with 1 mL of 4% autoclaved alginate solution so that the OD₆₀₀ for the final bacterial solution was 2.0. To make the bacteria-hydrogel biofilm, we added 11 μL of this suspension to a polydimethylsiloxane (PDMS) cavity (diameter 2.5 mm, depth 1200 μm), which was placed on a glass slide (2 × 3 cm), as shown in Figure 3A. The substrate was kept undisturbed for 20 min to form a hydrogel inside the cavity and the hydrogel was then further cured for 1 h in 1% CaCl₂ solution at 4 °C.

pH mapping on *S. mutans* alginate gel biofilm (*S. mutans* biofilm) by SECM.

The hydrogel-encapsulated bacterial biofilm was prepared as described in the previous section. The petri dish containing the biofilm was then put on an SECM stage, as shown in Figure S1A. AS solution with 1 mM ferrocenemethanol was later added to the dish and a PAC was performed with a pH microprobe by applying +0.4 V vs Ag/AgCl to the microprobe (Figure S1B). When the distance of the probe was fixed at 50 μm above the

biofilm, the solution was replaced with a solution of AS only and the temperature controller was turned on to increase the temperature of the solution inside the dish to 37 °C. Sucrose was added to the AS solution to a final concentration of 30 mM and the pH microprobe was connected to SECM through a high-impedance unit (EA Instruments) for potentiometric pH measurement. The current vs. time response was recorded at the pH sensor at 0.0 V vs Ag/AgCl, which was further converted to potential vs. time to estimate the change in pH on the biofilm surface (using the post-SECM experiment pH sensor calibration). Later, the z-direction pH profile was measured by moving the sensor probe from 50 to 1000 µm above the biofilm with the scan speed of 6 µm/s (Figure 3B). To study the change in pH profile above the *S. mutans* biofilm in the presence of sucrose in AS at 37 °C, SECM imaging experiments were carried out by using pH probe. pH probe was fixed at 60 µm above the biofilm to scan 3 mm X 3 mm area of a substrate to incorporate the entire 1 mm dia. biofilm. The data acquisition time for the entire 3 mm X 3 mm image area was approximately ~3 h with the scan speed of 30 µm/s (~10,000 pixels). Later the potentiometric (mV) image was converted to pH using appropriate calibration curve to obtain the final 3D pH profile above the biofilm (Figure 3D). Similarly, for biofilm morphology imaging experiment, the current (nA) image data was converted to height (µm) of the biofilm to obtain a final 3D biofilm topographical image. In this case, SECM image was recorded in the presence of 1 mM ferrocenemethanol in AS at 23 °C using pH probe with the same imaging parameters (Figure 3C).

pH and H₂O₂ mapping above dual-species (*S. mutans*-*S. gordonii*-*S. mutans*) biofilm.

The dual-species biofilm was prepared as described in the previous section. The detailed schematics of the *S. mutans*-*S. gordonii*-*S. mutans* biofilm are shown in Figure 4A. To quantify the change in local pH that was due to the lactic acid-producing *S. mutans* species, we used a dual-function SECM pH microprobe to map the distribution of the pH above the entire dual-species biofilm. The sample was placed on the SECM stage with AS solution that contained 1 mM ferrocenemethanol, as shown in Figure S1A, and the pH microprobe was then fixed 50 µm above the biofilm by recording an amperometric probe approach curve (PAC) above the biofilm in 1 mM ferrocenemethanol in AS solution (Figure S1B). Later, the solution was replaced with AS solution containing various amounts of glucose and sucrose and the temperature was adjusted to 37 °C. Initially, the pH probe was held at 50 µm above the *S. mutans* biofilm to measure the local change in pH. Later, a series of x-direction scans (scan speed 6 µm/s) were obtained by using the pH probe at 150 and 1000 µm above the *S. mutans*-*S. gordonii*-*S. mutans* biofilm. Z-direction pH scans (scan speed 6 µm/s) were also performed above the *S. gordonii* and *S. mutans* biofilms. The local H₂O₂ concentration at 50 µm above the *S. gordonii* biofilm surface in a dual biofilm was measured in the presence of 1 mM glucose and 30 mM sucrose in AS solution (pH 7.2 and 6.0) at 37 °C. Additional x-direction scans were performed at 150 µm and 1000 µm above the dual-species biofilm.

Fluorescent pH imaging of dual-species biofilm.

To estimate the bottom and top pH of *S. mutans*-*S. gordonii*-*S. mutans* biofilm, we performed fluorescence confocal imaging on Sm-Sg-Sm alginate gel biofilm with a LysoSensor™ Yellow/Blue dextran pH probe (pH molecular probe). Biofilm samples were prepared from *S. gordonii* and *S. mutans* grown for 12 h with a 2 µM pH molecular probe in

BHI media in 5% CO₂ at 37 °C. *S. mutans*-*S. gordonii*-*S. mutans* gel biofilm was made inside a PDMS mold glued to a micro cover glass (VWR, 24×50 mm, thickness 0.17–0.25 mm). The conjugated labeled dextran was incorporated in the exopolysaccharide matrix during bacterial growth^{32,33} and hence uniformly distributed in an alginate gel matrix during the formation of the gel biofilm. This molecular probe shows dual-emission spectra (maximum fluorescence emission at 452 nm and 521 nm) and the intensities of emissions depend on the pH. To observe changes in pH at the bottom and top of the gel biofilm, the sample was exposed to 1 mM glucose and 30 mM sucrose in AS (pH 7.2) at 37 °C for 2 h. Fluorescence intensities of two emission wavelengths and the ratio of fluorescence intensities at the bottom and top of the biofilm were collected by recording multiple snapshots (on the surface of Sg and Sm in dual biofilm) with a Zeiss LSM 780 NLO confocal microscope equipped with a multiphoton laser source with a 10X objective. With a two-photon laser source, the biofilm was excited at 760 nm and the emission was collected by using two channels of the LSM BiG detector as non-descanned detectors: one at 420–480 nm and the other at 500–550 nm. Fluorescence intensity was analyzed with 64-bit ZEN 2012 SP1 (Black Edition) software. To convert intensity ratios to pH values, we generated a calibration curve of intensity ratio vs. pH³² for the known pH (ranges from 4.5 to 7.2) of AS solution with a 2-μM pH molecular probe, as shown in Figure S5 (details regarding the calibration curve of intensity ratio vs. pH can be found in the Supporting Information (Figure S5)).

Genetic studies.

Overnight cultures of *S. gordonii* grown in BHI at pH 7.0 were inoculated 1:30 in fresh BHI at pH 7.0. Cultures were grown to mid-log phase (OD₆₀₀~0.4) at 37 °C, 5% CO₂. Cultures were centrifuged at 4000 rpm for 10 min. Pellets were resuspended in either BHI at pH 7.0 or BHI at pH 6.0 for continued growth. Cultures were sampled at 30, 60 and 120 min and RNA was extracted (details about RNA isolation, cDNA synthesis, and real-time PCR are given in the Supporting Information, Figure S7).

RESULTS AND DISCUSSION

Electrochemical characterization of H⁺-ISME (SECM pH probe).

Potentiometric characterization: The potentiometric response of the pH microprobe in universal buffer, BR buffer, and AS is shown in Figure 1B and Figure S2A. The sensor showed a broad dynamic range from pH 12 to pH 4 in BR buffer with a slope of 53.1 ± 4 mV/pH (Figure S2A) and from pH 10 to pH 4 in universal buffer with a slope of 60.4 ± 2 mV/pH (Figure 1B, black markers). Because we developed this sensor to study the metabolic activity of a lactic acid-producing oral microbe, *S. mutans*, in a biologically relevant environment, we also checked its performance in the AS solution ranging from pH 7.2 to pH 4. Figure 1B (red markers) shows the calibration curve in AS with a slope of 57.7 ± 3 mV/pH at 23 °C. The slope or the sensitivities and detection limit of the pH probe remained constant with and without oxygen (Figure 1C), confirming its insensitivities to variable amounts of oxygen, which is essential in an experiment with oxygen-consuming bacteria. In addition, the calibration curve of the pH sensor in the presence of 80 μM H₂O₂ (Figure 1C) confirmed that the sensor is not potentiometrically affected by the presence of

varied concentrations of H_2O_2 . We also tested the effect of applying potential to the sensor for a period of 30 min as an approach curve was performed by applying +0.4 V. We found that the slope remained consistent before and after the amperometric approach curve experiment (Figure S2B). The pH probe also showed a fast response time of <0.5 s (Figure 1D) and was thus suitable to use as an SECM pH probe to scan the x-y-z plane without distorting or compromising the pH chemical image.

Selectivity of any ion-selective electrode is an important parameter, as it allows detection of the analyte ion in the presence of interfering ions and is even more important in our live biofilm sample and complex matrix solution. Hence, the selectivity coefficient for the pH microsensor was estimated by the fixed interference method (Table 1).^{34,35}

Amperometric characterization: The competency of the sensor used in recording the PAC in SECM experiments was later confirmed by amperometric characterization of the sensor in a redox mediator, RuHex (Figure 2). We applied -0.35 V vs. Ag/AgCl (reduction potential of RuHex) to the pH probe and the SWV (Figure 2B and 2C) and i-t response was obtained with an increasing concentration of RuHex from 0.5 mM to 2.5 mM in 0.1 M KCl solution. Figure 2C indicates that the pH probe showed a linear relation between the RuHex concentration and the reduction current and thus can potentially be used as an amperometric probe to detect other redox molecules. The cyclic voltammogram obtained at the pH probe in 1 mM RuHex in 0.1 M KCl showed a steady-state voltammogram (Figure 2A), confirming that the probe can be used to perform a steady-state amperometric approach curve to fix the probe-substrate distance. However, the observed steady-state reduction current is higher than the theoretical value because of the higher electroactive surface area of the carbon paste. At the same time, we observed a kinetics limitation of this carbon-based pH electrode in the reduction of RuHex, as confirmed by the shift in negative potential by 130 mV compared with that of the Pt electrode (Figure 2A and 2B). This is not surprising, given that our carbon-based pH electrode contains 3% PVC for stabilizing the ionophore and related components such as plasticizers. We then further conducted a PAC by using this SECM pH probe on an insulating petri dish substrate that fit with the theoretical negative feedback approach curve (Figure 2D). This indicates that our newly developed pH sensor can be useful in SECM experiments to study the local change in pH above the bacterial biofilm.

pH mapping above *S. mutans* biofilm with the pH microprobe.

After confirming the Nernstian behavior of our newly developed 25- μm diameter carbon-based pH sensor, we put it to the test as an SECM sensor probe to quantitatively map the local pH above the lactate-producing *S. mutans* biofilm. The schematic experimental setup is shown in Figure 3A. The z-directional pH map from 50 μm above the biofilm to 1000 μm in bulk solution is shown in Figure 3B. The corresponding z- direction H^+ concentration profile estimated from the data shown in Figure 3B is given in the Figure S8A. After addition of 30 mM sucrose above the biofilm in an AS solution of pH 6.0 at 37 $^\circ\text{C}$, the pH at the biofilm surface dropped from 6.0 to 5.2 and the low pH profile was extended only up to 200 μm above the biofilm, which gradually increased to pH 6.0 within 600 μm above the biofilm in bulk solution. These results indicate that *S. mutans* creates a low pH zone only 200 μm above the biofilm and maintains a distinct acidic microenvironment compared with that in

bulk solution. Because our developed pH microprobe showed a fast response (<0.5 s), we used it for 3D pH mapping above the biofilm. Figure 3D shows a 3D pH image recorded on the 1-mm *S. mutans* gel biofilm. The pH at the biofilm surface dropped from 6.0 to 5.4 and the pH profile extended in both the x and y directions by about 500 μm and created a low pH zone surrounding the *S. mutans* biofilm. However, there are no such features observed in the 3D morphological image (Figure 3C) recorded on the *S. mutans* hydrogel biofilm with pH microprobe in the ferrocenemethanol redox mediator. Thus, our newly developed solid-state, carbon-based pH microprobe is capable of high-resolution pH mapping above the live bacterial biofilm in order to study the metabolic activity of the microbes under physiological conditions. Further, we used this sensor to study the metabolic interaction of H_2O_2 -producing *S. gordonii* and acid-producing *S. mutans* in a dual-bacteria biofilm.

Effect of local pH on bacterial metabolic activity: an SECM study on the dual-species, *S. gordonii* and *S. mutans* biofilm.

After quantifying the local pH from lactic acid production by the *S. mutans* biofilm, we further extended our studies to the effects of this low pH profile on another closely situated bacterial species, H_2O_2 -producing *S. gordonii*. We thereby aimed to explore the interdependence of H_2O_2 and lactic acid production by *S. gordonii* and *S. mutans* in the presence of two different pH conditions, pH 6.0 and pH 7.2, which are at the edge and midpoint, respectively, of the buffering range of AS. To obtain a quantitative surface pH map across the two species, we used our solid-state, carbon-based pH microprobe (Figure 1A) as an SECM tip (Figure 4A). We observed that the local pH and its z-directional profile from 50 to 1,000 μm above *S. gordonii* in the *S. mutans*-*S. gordonii*-*S. mutans* biofilm changed from 5.3 to 6.0 in the presence of 30 mM sucrose in an AS solution of pH 6.0 (Figure 4D, red solid curve). However, no change in pH value was observed in a similar z-directional pH mapping experiment with an AS solution of pH 7.2 (Figure 4D, red dotted curve). A different trend was observed for H_2O_2 production by *S. gordonii* in a dualspecies biofilm. We observed that the local H_2O_2 concentration was suppressed to 12 ± 5 μM for both pH solutions (6.0 and 7.2) (Figure 4D, black curves). Figure S8B shows the comparison of the changes in the H^+ concentration and H_2O_2 concentration from the surface of the *S. gordonii* in dual biofilm to the bulk in both pH 6.0 and 7.2 AS solution. These observations suggest that the chemical environment above the *S. gordonii* region was being influenced by the surrounding *S. mutans* lactic acid production, which also affected the H_2O_2 -producing capabilities of *S. gordonii*. However, the ability of *S. mutans* to change the surface pH directly depends on the buffering capacity of the added solution. Hence, to elucidate the effect of the pH profile produced by *S. mutans* biofilm in an x-y plane, we used our pH probe to quantitatively map this parameter at 150 μm above the entire *S. mutans*-*S. gordonii*-*S. mutans* biofilm (Figure 4B). We observed that the pH above this biofilm was about 5.4 and that its profile extended along the entire dual biofilm in the presence of a pH 6.0 bulk (~ 500 μm above the biofilm) solution, whereas the surface pH remained unchanged in the presence of a pH 7.2 solution. This is an important finding, as it indicates that AS solution can withstand acid-producing bacteria as long as its buffering capacity remains intact; however, when the AS buffer is at pH 6.0, it can no longer maintain its buffering capacity. The lactic acid produced by *S. mutans* biofilm is enough to titrate the AS solution, resulting in the observed pH shift. Nonetheless, H_2O_2 production is still suppressed, suggesting the

possibility of low pH at the bottom of the biofilm. This observation can only be explained by the buffering capacity of the AS solution, which mimics the buffering capacity of human saliva.^{36,37} Another factor that played an important role beyond the local buffering capacity was the extension of the pH profile in the x-y and z directions. From Figures 4C and 4D, it is obvious not only that the amount of H₂O₂ was suppressed, but also that the extension of the H₂O₂ profile was limited above *S. gordonii*, whereas the pH profile extended ~1000 µm over the two species (Figure 4B). This finding indicates that *S. gordonii* dominated the landscape by producing a high amount of H₂O₂, whereas *S. mutans* slowly titrated the solution's buffering capacity via lactic acid production. This ultimately allowed *S. mutans* to overcome the H₂O₂ effect by extending a low pH profile over *S. gordonii*. Interestingly, the pH-dependent reduction in H₂O₂ production was not immediately effective.

pH mapping at the top and bottom of the dual-species biofilm by confocal fluorescence microscopy.

Since there was no surface pH shift, but *S. gordonii* H₂O₂ production decreased in a dual-species biofilm setup, we performed fluorescent-based pH mapping of the biofilm to confirm whether pH at the bottom of the biofilm shifted. As observed in Figure 5A, there was no significant change in surface pH, whereas the pH at the bottom of the biofilm shifted from 7.2 to 5.3. As discussed in the previous section, the SECM study quantified the local pH above the biofilm. However, we also wanted to quantify the pH at the bottom of the biofilm to elucidate whether the pH shift happened even when the surface pH did not shift, for example, for pH 7.2 buffer. This is important for a small confined space such as micro cracks, which are a common phenomenon in the dental environment.

Differential H₂O₂-producing *spxB* gene expression.

The pH-dependent decrease in the extracellular H₂O₂ concentration was further confirmed phenotypically by using indicator plates for H₂O₂ production. *S. gordonii* cells were inoculated on pH-adjusted (pH 7.0 and pH 6.0) Prussian blue agar. This indicator agar allows for the detection of bacterial H₂O₂ production by forming a blue precipitate of Prussian blue in the presence of H₂O₂. After the cells were grown overnight in the presence of 5% CO₂, the production of H₂O₂ on the pH 6.0 plate was markedly reduced in comparison to that on the pH 7.0 plate, while bacterial growth was not affected (Figure 5B).

The pH-dependent reduction of extracellular H₂O₂ could be the result of inactivation of the pyruvate oxidase (*SpxB*) activity, or a decrease in the expression of the *spxB* gene. To determine whether a change in pH from 7.0 to 6.0 alters *spxB* expression, we grew cells to mid-logarithmic phase at pH 7.0, harvested them by centrifugation, and resuspended them in BHI medium adjusted to either pH 6.0 or pH 7.0. Expression of *spxB* was determined 30, 60, and 120 min after the shift to pH 6.0 and was normalized to the expression at pH 7.0. In agreement with the observation that the local H₂O₂ concentration in a pH 6.0 solution was the same as that of a pH 7.2 solution, we did not observe a significant reduction in *spxB* expression over the first 60 min. However, 120 min after the shift, we observed a significant reduction in *spxB* expression (Figure 5C). These results are in agreement with the reduction in H₂O₂ concentration determined by SECM and the phenotypic observation of H₂O₂ production on indicator plates.

Proposed metabolic interaction model.

Our time-resolved SECM experiments demonstrate that *S. gordonii* is initially able to counteract a potential pH challenge with a moderate increase in *spxB* expression and H₂O₂ production. This reflects a situation during a normal meal with moderate carbohydrate intake. *S. gordonii* is able to balance homeostasis by inhibiting the growth or metabolic activity of acid producers, thus allowing for stabilization of the biofilm species composition. However, longer exposure to a lower pH severely affects competitive H₂O₂ production and therefore favors a shift in microbial composition to aciduric bacterial species, allowing for the development of caries. This result is a clear indication that the metabolic profile of the local chemical environment can have a significant effect on bacterial metabolic activity and has consequences for the pathogenicity of one species over another. This phenomenon might be exaggerated inside a small confined volume, such as dental crevices, where the buffering capacity of the saliva cannot outlive the *S. mutans* acid attack. This prediction is confirmed by in situ pH mapping at the top (pH 7.2) and the bottom (pH 5.5) of the dual biofilm (Figure 5A) with confocal fluorescence microscopy. The observed reduction in the ability of *S. gordonii* to produce competitive amounts of H₂O₂ is caused by reduced transcription of the *spxB* gene (Figure 5B and 5C). We demonstrate here that a shift from pH 7.0 to pH 6.0 results in a transcriptional response, leading to a two-fold reduction in *spxB* expression and a decrease in secreted H₂O₂, which influences the competitive ability of *S. gordonii* in interspecies interactions with *S. mutans*. As shown previously, H₂O₂ is the only antimicrobial substance produced by *S. gordonii* that is able to inhibit the growth of *S. mutans*. An acidogenic and aciduric microorganism, *S. mutans* has the ability to inhibit competing species such as *S. gordonii* by producing lactic acid.

CONCLUSION

We successfully demonstrated the application of our newly developed solid-state, carbon-based pH microprobe to map the 3D chemical environment above an acid-producing *S. mutans* biofilm. With the 60% conducting Vulcan carbon in the sensor cocktail, the sensor showed a promising response in amperometry as well as in potentiometry. The sensor showed Nernstian response with a fast response time (< 0.5 s) and thus provided high-resolution pH images in SECM experiments. With this pH microprobe, we are also able to study metabolic interactions between two oral bacterial species, *S. mutans* and *S. gordonii*. We discovered that the production of H₂O₂ by *S. gordonii* was suppressed by the immediate presence of acid-producing bacteria, *S. mutans*. The results suggest that H₂O₂-producing bacteria such as *S. gordonii* are dominant while the buffering capacity of the saliva is still valid (~pH 6.0–7.2), but that *S. mutans* gradually takes over by decreasing the local pH to 5.0 or less through lactic acid production. Elucidation of the metabolic interactions of these microbes in 3D space—especially the larger pH profile distribution over that of H₂O₂ and thus their corresponding roles in determining the pathogenicity of *S. mutans* in the presence of beneficial *S. gordonii*—is made possible only by SECM. The unique capabilities of SECM, combined with a micro-sized ion-selective electrode or pH probe, in quantitative chemical mapping with high spatial resolution makes it a powerful tool in the investigation of these bacterial metabolic interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by the National Institute of Dental and Craniofacial Research, NIH Grant # R21DE025370 (Dipankar Koley) and NIH Grant # DE021726 (Jens Kreth). Authors would like to acknowledge the Center for Genome Research and Biocomputing at Oregon State University for Confocal and Two-Photon Excitation Microscopy facility (NSF # 1337774). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Figure 1. Potentiometric characterization of the pH sensor:

(A) Schematics of H⁺ ion selective membrane electrode (H⁺ ISME or pH microprobe). Here, DOS represents dioctyl sebacate. (B) Calibration of pH microprobe in universal buffer from pH 10 to pH 4 and artificial saliva (AS) solution from pH 7.2 to 4.0. (C) pH microprobe calibration in artificial saliva in the presence of the redox mediator RuHex, ferrocenemethanol and with and without O₂ in AS solution. (D) Response time of the pH sensor.

Figure 2. Amperometric characterization of pH sensor:

(A) Cyclic voltammogram recorded at the pH sensor in N_2 -purged 1 mM RuHex in 0.1 M KCl. (B) Square wave voltammetric (SWV) curves recorded in various concentrations of RuHex in a 0.1 M KCl solution at the pH sensor. (C) Linear relation of current obtained from the SWV and i-t curves with various concentration of RuHex. (D) PAC recorded at the glass surface with a pH probe.

Figure 3. 3D pH mapping above *S. mutans* biofilm:

(A) Schematic of *S. mutans* bacteria gel biofilm substrate and pH microprobe used in SECM experiments. (B) Z-directional pH profile mapping from 50 μm above the *S. mutans* biofilm to 1000 μm in the bulk solution after addition of 30 mM sucrose in artificial saliva (pH 6.0) at 37 °C. Scan speed was 6 μm/s. (C) 3D morphological image of the *S. mutans* bacteria gel biofilm substrate recorded 60 μm above the biofilm with pH microprobe (scan speed, 30 μm/s) in 1 mM ferrocenemethanol in AS (pH 7.2) at 23 °C. (D) 3D pH image recorded 60 μm above the biofilm with pH microprobe (scan speed, 30 μm/s) after addition of 30 mM sucrose in artificial saliva (pH 6.0) at 37 °C.

Figure 4. Metabolic activity of *S. gordonii* and *S. mutans* in a dual biofilm in artificial saliva.

(A) Schematic of dual-bacteria gel biofilm substrate: *S. gordonii* surrounded by *S. mutans* (*Sm*) used in SECM experiments. White dashed line represents the x-direction probe scan curve recorded with H_2O_2 and pH ultramicrosensors to measure the H_2O_2 and pH profile on the dual biofilm. (B) X-direction pH profile 150 μm above the dual-bacteria biofilm in the presence of glucose (G), sucrose (S), and glucose+sucrose (G+S) at pH 6.0 and 7.2. Glucose and sucrose concentrations are 1 and 30 mM, respectively. (C) X-direction H_2O_2 profile 150 μm above the dual-bacteria biofilm in the presence of G and G+S at pH 6.0 and 7.2. (D) Z-direction H_2O_2 and pH profile from 50 μm above *S. gordonii* in dual-bacteria biofilm to 1000 μm above in the bulk solution in the presence of G+S at pH 6.0 (solid lines) and 7.2 (dashed lines).

Figure 5. Confocal fluorescence pH mapping and *spxB* gene expression study.

(A) Confocal fluorescence pH mapping at the top and bottom of dual (*S. mutans*-*S. gordonii*-*S. mutans*) biofilm (average of two sets of experiments) after exposure to glucose + sucrose in pH 7.0 artificial saliva for 3 h at 37 °C (n = 3 for each set). (B) Representative image (n = 3) of H₂O₂ production of *S. gordonii* during aerobic growth at pH 7 and pH 6. (C) *spxB* gene expression in *S. gordonii* at pH 7 and after a shift from pH 7 to pH 6 determined at the indicated time intervals; mean and standard deviation are shown (n = 3). One-way analysis of variance; * p < 0.046.

Table 1:

Interference study of pH microprobe

Interfering ion, A	log K_H^+ , A
	Fixed interference method
Na ⁺	< -11.42
K ⁺	< -10.33
Ca ²⁺	< -11.06
Mg ²⁺	< -8.51