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An improved Severinghaus-type carbon dioxide microelectrode for use in biofilms

Haluk Beyenal [a], Catherine C. Davis [b], Zbigniew Lewandowski [a,c,*]

[a] *Center for Biofilm Engineering, P.O. Box 173980, Room 366 EPS, Montana State University, Bozeman, MT 59717-3980, USA*
[b] *Clinical Microbiology, PS&RA FemCare, The Procter & Gamble Company, WHTC, 6110 Center Hill Avenue, Cincinnati, OH 45224, USA*
[c] *Department of Civil Engineering, Montana State University, Bozeman, MT 59717, USA*

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Abstract

Severinghaus-type carbon dioxide microelectrodes with a tip diameter of less than 20 μm were constructed using anodically grown iridium oxide film (AIROF) as the internal pH sensor. The AIROF, which was formed at the tip of the iridium wire by cyclic voltammetry in diluted sulfuric acid, showed a super-Nernstian response to pH changes, with a slope between 65 and 80 mV/pH. Therefore, our microelectrodes were more sensitive to changes in carbon dioxide concentration than the previously described microelectrodes, which used liquid ion exchanger (LIX) pH membranes and had a Nernstian response of 59 mV/pH. When calibrated, our microelectrodes showed 65–76 mV per decade of μatm pCO_2, and were more sensitive than the microelectrodes using LIX membranes, which showed 57 mV per decade of μatm pCO_2. AIROF electrodes were serviceable after 1 month of storage, showing only a modest potential drift of 0.03 ± 0.01 mV/h. Their utility was demonstrated by measuring CO_2 concentration profiles in *Staphylococcus aureus* (MN8) biofilms.

Keywords: Carbon dioxide; Microelectrode; Iridium oxide; Biofilm

1. Introduction

Microelectrodes are indispensable tools for evaluating chemistries in biofilms at the microscale: they are used to measure concentration profiles of dissolved oxygen, pH, hydrogen sulfide, ammonia, and other dissolved gases and ions. The design of the microelectrodes usually imitates the design of the larger electrodes, except for the tip, which is smaller, typically several micrometers. The small tip of the microelectrode is needed to prevent damaging biofilm structure during measurements.

The carbon dioxide electrode was first developed by Severinghaus and Bradley [1], and a miniaturized version of that electrode was constructed some 20 years later by Caflisch and Carter [2]. Since carbon dioxide is an important product of biochemical reactions (e.g. aerobic respiration), much research effort has been devoted to constructing and using CO_2 microelectrodes [3]. Fig. 1 shows a classical CO_2 microelectrode that uses an internal pH sensor with a liquid ion exchanger (LIX) membrane. In this paper, we describe an improved Severinghaus-type CO_2 microelectrode that uses an iridium oxide pH sensing element instead of an LIX membrane. This type of microelectrode has a higher sensitivity to carbon dioxide than the microelectrodes with internal pH sensors with LIX membranes, and it is easier to manufacture.

Fig. 1 shows the CO_2 microelectrode with an internal pH microsensor using a liquid ion exchange membrane [3–5]. The pH sensor is located within the shaft of the electrode behind a gas permeable membrane. To be useful in biofilm research the tip of the external sensor has to be smaller than 20 μm. Constructing CO_2 microelectrodes having an internal pH sensor and small tip diameters is difficult, and the success ratio is lower than that with other microsensors. For example when we construct dissolved oxygen microelectrodes, about 7 out of 10 are usable. However, when we construct CO_2 microelectrodes with LIX pH internal sensor only 1 out of 10 is expected to be usable (other have non-reproducible calibration curves, exhibit high shift in potential, or do not respond to carbon dioxide for unknown reasons). Although the reasons for these difficulties are mostly unknown, some may be traced to the need of using small tip diameters. Perhaps because of that some biofilm researchers use larger tip diameters, e.g. Zhao and Cai [3] used CO_2

* Corresponding author. Tel.: +1-406-994-5915; fax: +1-406-994-6098.
E-mail address: zl@erc.montana.edu (Z. Lewandowski).

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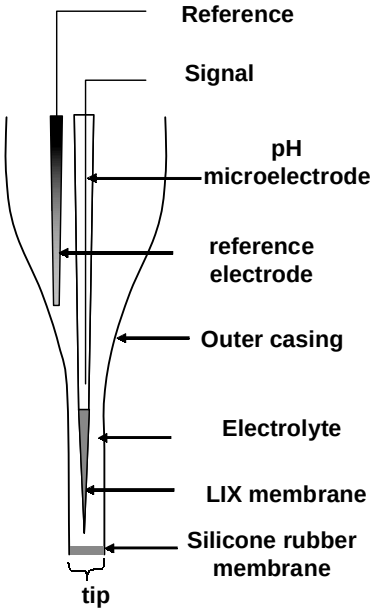

Fig. 1. Diagram of the Severinghaus-type microelectrode constructed by de Beer et al. [4] and Zhao and Cai [3].

microelectrodes with tip diameters between 50 and 300 μm and Komada et al. [6] used a CO_2 microelectrode with 250 μm tip diameter. The life-time of CO_2 microelectrodes with the internal pH sensor using LIX membrane is short because LIX slowly leaks into the shaft. In our measurements, these microelectrodes exhibited potential drift ranging from a few mV/h to 10 mV/h, and a short life-time, several hours to a few days during routine measurement. These problems are all related to the pH sensor; potential drift is likely caused by the leakage of the LIX membrane, and the short life-time is related to the short life-time of the pH microsensors [7].

Another common problem with CO_2 microelectrodes is the long response time, measured in minutes, which is probably caused by the fact that these microelectrodes have two membranes, a gas-permeable membrane at the tip of the external sensor and an LIX membrane at the tip of the internal pH sensor. It is therefore expected that the time to equilibrate the chemistries in the two internal electrolytes may be long.

Most of the difficulties with using carbon dioxide microelectrodes can be traced to the pH internal sensor, which prompted us to design a carbon dioxide micro electrode that uses an iridium oxide pH sensor. We are not alone in this pursuit for better CO_2 electrodes: a new approach to constructing CO_2 electrodes was attempted by Suzuki et al. [8], who constructed a micromachined CO_2 electrode using anodically grown iridium oxide film (AIROF) as the pH-sensing element. The device was rather large, 1.5 mm wide, 13 mm long and 0.9 mm thick, certainly larger than those we use to study biofilms. However, the idea of using an iridium oxide pH sensor was appealing because these sensors are known to have a faster response to pH than those using LIX, exhibit less drift and have a longer life-time [9], and because we routinely constructed and use iridium oxide pH microelectrodes in our laboratory [10]. We hypothesized that carbon dioxide microelectrodes with an internal iridium oxide pH microsensors should last longer and respond faster because they do not have the LIX membranes, and that they should be more sensitive to carbon dioxide because AIROF pH microelectrodes exhibit a super-Nerstian response [10], explained by the mechanisms predicting on the average 1.5 protons transferred per one electron with a slope of 90 mV/pH [8–14].

$$2[IrO_2(OH)_2 2H_2O]^{-2} + 3H^+ + 2e^- \Leftrightarrow [Ir_2O_3(OH)_3 3H_2O]^{-3} + 3H_2O \quad (1)$$

2. Operational principles of CO_2 microelectrodes

During the measurements, carbon dioxide dissolved in the external solution, $CO_2(g)$, diffuses through the silicone rubber membrane and reaches the internal solution, where part of it dissolves and form $CO_2(aq)$:

$$CO_2(g) \Leftrightarrow CO_2(aq) \quad (2)$$

The dissolved carbon dioxide, $CO_2(aq)$, undergoes hydration and forms carbonic acid, H_2CO_3 [8].

$$CO_2(aq) + H_2O \Leftrightarrow H_2CO_3 \quad (K_H = 0.034) \quad (3)$$

where K_H is Henry's constant.

The carbonic acid, H_2CO_3, dissociates in two steps:

$$H_2CO_3 \Leftrightarrow H^+ + HCO_3^- \quad (K_1 = 1.72 \times 10^{-4}) \quad (4)$$

$$HCO_3^- \Leftrightarrow H^+ + CO_3^- \quad (K_2 = 5.59 \times 10^{-11}) \quad (5)$$

where K_1 and K_2 are the first and second dissociation constants for H_2CO_3. The relationship between $[H^+]$ and pCO_2 can be calculated from Eqs. (3)–(5) [8]:

$$[H^+]^3 + [NaHCO_3][H^+]^2 - (K_1 K_H pCO_2 + K_w)[H^+] - 2K_1 K_2 K_H pCO_2 = 0 \quad (6)$$

where K_w is the ionic product of water ($=10^{-14}$).

The internal solution of carbon dioxide electrodes is composed of carbonic acid and bicarbonate, and its pH can be calculated using the Henderson–Hasselbach equation:

$$pH = pK_a + \log\frac{[HCO_3^-]}{[H_2CO_3]} \quad (7)$$

From Eq. (7), the concentration of carbonic acid, which for all practical purposes is equal to the concentration of carbon dioxide, can be estimated:

$$\log[H_2CO_3] = pK_a + \log[HCO_3^-] - pH \quad (8)$$

The internal electrolyte is prepared in such a way that the concentration of bicarbonate ions is exceedingly large, which makes the change in bicarbonate concentration,

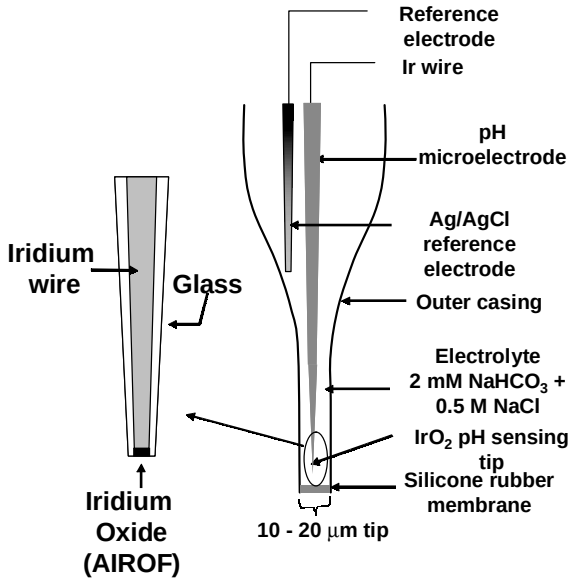

Fig. 2. The microelectrode consists of (1) internal AIROF pH microelectrode, (2) outer casing, (3) electrolyte solution, and (4) reference electrode.

$[HCO_3^-]$, negligible compared to the pH changes, and therefore:

$$\log[H_2CO_3] \cong \text{constant} - \text{pH} \quad (9)$$

In summary, by preparing the internal electrolyte with an excess of bicarbonate, the pH in the internal electrolyte solution is kept proportional to the activity of carbon dioxide in the external solution, which makes the electrode sensitive to carbon dioxide, even though it really measures pH.

Replacing the internal pH sensor with iridium oxide (Fig. 2) does not change the principle of action; it only changes the performance.

3. Experimental

3.1. AIROF pH microelectrode

The preparation of AIROF is thoroughly described in the literature [9–12]. We followed the procedure for making Ir/IrO_2 pH microelectrodes developed by our research group [10]; the following sections give a short description of this and other procedures employed in constructing the microelectrode.

3.1.1. Tapering iridium wire

The 75 μm diameter iridium wire (99%, Engelhard) is cut into sections of 3–5 cm and the sections are washed with distilled water. To taper the tips to the appropriate diameters, each section is anodically dissolved in 0.5 M H_2SO_4 until the tip is between 2 and 10 μm. In this process, we apply 10 V dc (Tektronix PS2526 power supply) between the iridium wire and a graphite counter electrode (Aldrich, catalog number: 49654-5, 3 mm in diameter).

3.1.2. Making glass capillary

To make glass capillaries, we use glass tubes of 1.5 mm outer diameter (Corning 8161). We wash the tube first with acetone, then with distilled water and then with acetone again, and allow the acetone to fully evaporate. The glass tube is cut into 10 cm sections using a diamond pen, and these sections are then pulled over the flame of a propane torch to form capillaries. The capillaries are broken in the middle to separate the two sides of the pulled section, and stored in a dust-free place.

3.1.3. Covering the iridium wire with glass

The tapered end of the Ir wire is inserted into the glass capillary. The capillary, with the iridium wire inside it, is hung in a microelectrode puller (Stoelting, model 51217) with the tapered tip up. While manufacturing microelectrodes, we typically do not use the entire setup of the Stoelting micropipette puller, only its heating element. An M-shaped electrical wire is used to apply heat, about 1.5 cm below the tapered iridium wire tip inside the capillary. When the heat is applied, the glass melts, flows down slowly, and covers the iridium wire with a thin continuous layer.

3.1.4. Grinding the tip of the capillary

The glass covering the iridium wire extends past the tip of the iridium wire, and this excess has to be removed to expose the tip of the wire. To expose the tip we use a diamond grinding wheel combined with a micromanipulator (Narishige, model EG-4). The surface of the grinding wheel and the tip of the iridium wire are continuously monitored using a COHU camera attached to a microscope (Infinity, model CFM, Erect Image) that is integrated with a TV monitor. The grinding stops when the excess glass is removed and the tip of the wire is exposed. The diameter of the exposed tip is typically several microns. The tip of the capillary is gently rinsed with distilled water to remove glass particles accumulated during grinding, then washed with acetone to remove grease that may cover the wire and change its electrical properties, and then washed with distilled water in a sonicator bath to remove the remaining glass debris from the tip.

3.1.5. Recessing the iridium wire

In the next step, the tip of the wire is recessed by a few microns into the glass capillary. The reasons for this are purely mechanical; it is known that AIROF adheres poorly to the metal surface [13]. Therefore, to improve mechanical properties of the tip and to ensure that the anodically formed iridium oxide will remain in position, the iridium wire exposed by grinding the excess glass is recessed into the microcapillary using anodic polarization in 0.5 M H_2SO_4 with a graphite counter electrode (Aldrich, catalog number: 49654-5, 3 mm in diameter). The chemical principle of the process is the same as that used to taper the tip of the wire, anodic dissolution of iridium wire in sulfuric acid, but this time only the tip of the wire is exposed to the acid solution.

To facilitate the process, we apply 5 V dc in 0.5 M H_2SO_4 using a Tektronix PS2526 power supply and a graphite counter electrode (Aldrich, catalog number: 49654-5, 3 mm in diameter). The process continues until the wire is recessed by a few microns. The procedure is completed by cleaning the tip of the wire in a sonicator, first with acetone and then with distilled water. The cleaning is completed by polarizing the wire cathodically for 10 min in 0.05 M Na_3PO_4 at −5 V dc against a graphite electrode (Aldrich, catalog number: 49654-5, 3 mm in diameter). During cleaning the solution is mixed using a magnetic stirrer to remove hydrogen bubbles from the tip. The presence of bubbles in the recessing tip disrupts the flow of current and interrupts the etching process. Finally, the micro electrode tip is rinsed with distilled water.

3.1.6. Growing iridium oxide films on the iridium wire

Iridium oxides are deposited on the tip of the wire by cyclic polarization in 0.5 M H_2SO_4 solution, with the iridium wire as the working electrode. A triangular voltage waveform is applied between −0.25 and +1.4 V (versus Ag/AgCl) at 0.15 V/s using an EG&G (PARC 273, Princeton, NJ) potentiostat/galvanostat with an Ag/AgCl reference electrode and a platinum counter electrode. The potential is swept for at least 4000 cycles, and the formation of the iridium oxide film (AIROF) is monitored by observing the shape of the voltammograms. This procedure is based on the methodologies outlined by VanHoudt et al. [10], Pickup and Birss [14], and Rand and Woods [12]. After the process is completed, the AIROF is aged in distilled water for 12 h, followed by aging in air for another 12 h, and the pH microelectrode is stored in a dust-free environment.

3.1.7. Making the outer case

The outer case is constructed from a glass Pasteur pipette (Fisher 13-678-20C). The pipettes are cut to the appropriate length to accommodate the iridium oxide pH microelectrodes, which are 7–10 cm long. The cut end of each pipette is fire-polished by inserting the pipette into the flame of a propane torch equipped with a thin nozzle, and keeping it there until the edge of the glass is smooth. To make the outer diameter of the sensor small enough to be useful in probing biofilms, the outer case is tapered down to a 10 μm tip diameter. To accomplish this, the pipette is suspended by its thin end in a micromanipulator and placed near the M-shaped heating element of the micropipette puller (Stoelting, model 51217). The pipette is lowered to the tapered region to start the necking down of the pipette. The heat is applied until the heating element glows red. When the glass begins to melt and flow, and the pipette begins to drop, the heat delivery is quickly stopped. The pipette is then pulled up to the next necked position. This sequence is repeated until a thin tip of the capillary is produced, approximately a few hundred μm in diameter. For the final thinning of the tip we use a thinner heating element, made of a 100 μm Pt wire, powered by a variac (Powerstat, type 116B, input voltage: 120, output voltage: 0–140, 10 A) and a transformer (Chicago Standard Transformer, Catalog number FMS-620. 6.3 V, 20 A, maximum 100 V). We repeat the procedure for thinning the tip, applying the heat and stopping the current flow through the heating element when the pipette begins to drop. Usually, just two applications of these procedures are enough to produce small tip diameters. Then, the tip of the outer case is broken under a microscope using 40 or 100 times magnification by jamming it against a glass ball made on the tip of a thin glass rod. Jamming the casing into the glass ball is continued until the tip of the outer case is between 10 and 20 μm in diameter. Then the sharp edges of the outer case's tip are gently smoothed by applying heat to the tip using the heating element made of a 100 μm Pt wire (as described above).

3.1.8. Applying the external membrane

The casing must be covered with a gas-permeable membrane, and we use silicone rubber membranes (ACE 11316, from ACE hardware store) for that purpose. To apply the silicone rubber membrane, the tip of the outer case is immersed into a drop of the silicone rubber deposited on the end of a thin glass rod. As a result, the silicone rubber is sucked into the tip by capillary action, filling approximately 5–10 μm of the tip. If the silicone rubber is too dense and does not fill the tip of the capillary, it can be diluted in acetic acid. The silicone rubber membrane is allowed to cure for at least 24 h.

3.1.9. Internal Ag/AgCl reference electrode

The carbon dioxide microelectrodes are equipped with the internal Ag/AgCl reference electrodes. We make these electrodes using a silver wire 7–10 cm long (0.25 mm diameter, Aldrich 32703-4). The wire surface is first cleaned using 600-grit sandpaper, rinsed with distilled water, then dipped in 3 M HNO_3, and again rinsed with distilled water. To apply the layer of silver chloride, the silver wire is anodically polarized by applying a voltage of 0.1 V dc overnight against a graphite electrode (Aldrich 49654-5) in a 0.1 M HCl solution. After chlorinization is completed, the Ag/AgCl wire is aged for 1–2 days in 0.1 M HCl to produce a uniform layer of silver chloride.

3.1.10. Electrolyte solution

The internal electrolyte solution is made of 2 mM $NaHCO_3$ (Fisher S233-3), 0.5 M NaCl (Fisher S-271-10) and 0.5 mg/ml carbonic anhydrase (Sigma C-3934).

When we expect that the electrode will be stored for a long time, we add 5 mg/l chloramphenicol (Fisher, BP901-100) to the electrolyte, to prevent microbial growth in the electrolyte.

3.1.11. Calibrating AIROF pH microelectrodes

Before the pH microelectrode is inserted into the casing, it is calibrated to make sure that it works properly. AIROF

pH microelectrodes are calibrated using a set of commercial buffer solutions between pH 4 and 10. We use a Keithley model 6517A electrometer, with a high input impedance of 200 TΩ, to measure potential between AIROF microelectrodes and a commercial saturate calomel electrode (SCE).

3.1.12. Assembling the components

The iridium oxide pH microelectrode and the outer case are assembled under a microscope using micromanipulators. Before inserting the pH microelectrode, a few drops of the internal electrolyte solution are added into the outer case using a syringe. It is common for an air bubble to reside stubbornly at the tip of the outer case. The air bubble between the silicone rubber and the electrolyte is removed by placing the outer case in a vacuum, which causes the electrolyte to boil. Once the air gap has been removed, the pH microelectrode is inserted into the casing. The tip of the pH microsensor is positioned 5–10 μm behind the silicone rubber membrane, glued with 5 min epoxy (industrial grade, from a hardware store), and filled with the electrolyte. Then, the Ag/AgCl reference electrode is inserted into the outer casing and fixed using the same 5 min epoxy. The top of the microelectrode is sealed with silicone rubber to prevent evaporation of the internal solution.

3.1.13. Calibrating the CO_2 microelectrodes

The CO_2 microelectrodes are calibrated before and after measurements in biofilms using CO_2 gas standards prepared in our laboratory by mixing CO_2 and N_2 at various ratios. The tip of the microelectrode (sensitive area) is immersed in the stirred distilled water, and the gas mixture is slowly fed into the water. The actual CO_2 partial pressure (P_{CO_2}) is calculated by correcting barometric pressure (P_B) using water vapor pressure (P_w):

$$P_{corr} = P_B - P_w \tag{10}$$

P_{CO_2} of the calibration gas is calculated as:

$$P_{CO_2} = \left(\frac{\%CO_2}{100}\right)_x P_{corr} \tag{11}$$

where ($\%CO_2$/100) corresponds to the fraction of CO_2 in the gas mixture.

3.2. Using the carbon dioxide microelectrode

The utility of the microelectrodes was demonstrated by measuring carbon dioxide concentration profiles in biofilms of *Staphylococcus aureus* (MN8). The biofilm was grown in a growth medium made of 1% glucose; 1/50 LB medium, saturated with air in a one-pass flow-through reactor at 37 °C; and 0.200 mM of dissolved oxygen. The reactor was inoculated with a 24 h-old culture of the organism, and the biofilm was allowed to grow in a quiescent state for 24 h. Flow of the growth media at 30 ml/h was then started and continued for 3 days. The reactor was operated in a custom-designed temperature-controlled chamber, and was mounted on an Olympus CK2 inverted microscope. The biofilm structure was monitored through a transparent window (cover slip) in the bottom of the reactor. This arrangement also allowed for monitoring the position of the tip of the microelectrode when carbon dioxide concentration profiles were measured.

To measure carbon dioxide concentrations, the electrodes were introduced into the reactor through a hole in the lid, which remained sealed by a rubber stopper during the operation. Just before the measurements, the rubber stopper was removed while the flow of liquid and gases continued. The microelectrodes were mounted on a micromanipulator (model M3301L, World Precision Instruments, New Haven, CT) equipped with a stepper motor (model 18503, Oriel, Stratford, CT) controlled by an Oriel model 20010 interface. Microelectrodes were introduced from the top of the reactor at an angle perpendicular to the biofilm. The micromanipulator was interfaced with a computer, and the microelectrode movement was facilitated by a controller (CTC-283-3, Micro Kinetics) with a positioning precision of 0.1 μm. Custom software was used to control and coordinate microelectrode movement, data acquisition, and real-time display of the concentration profile.

A small region of the glass cover-slip window was cleared so that the tip of the microelectrode could be observed using the inverted microscope. To locate the position of the bottom, the microelectrode was stepped down until it was approximately 2–4 μm from the glass bottom. We noted this position as the bottom of the biofilm. Then the microelectrode was removed and positioned above the top of a cell cluster we selected for measurements, and the CO_2 profile was measured at the middle of this cluster. Two more measurements were taken at the edges of the cell cluster.

4. Results and discussions

4.1. Calibrating AIROF pH microelectrodes

A typical pH microelectrode calibration is shown in Fig. 3. Freshly prepared microelectrodes typically showed a slope between 65 and 80 mV/pH after two days of aging. To test the effective life-time of the pH microelectrodes we stored five electrodes in the electrolyte solution for 1 month. Fresh electrodes had a slope of 72.3±7.7 mV/pH; microelectrodes aged for 1 month, 63.7 ± 5.1 mV/pH.

4.2. Calibrating CO_2 microelectrodes

The calibration curve is shown in Fig. 4. The lowest concentration of carbon dioxide we could detect was 150 μatm, which is slightly lower than that detected by Zhao and Cai [3], 250 μatm. In repeated experiments, the slopes of the calibration curves were between 65 and 76 mV per decade,

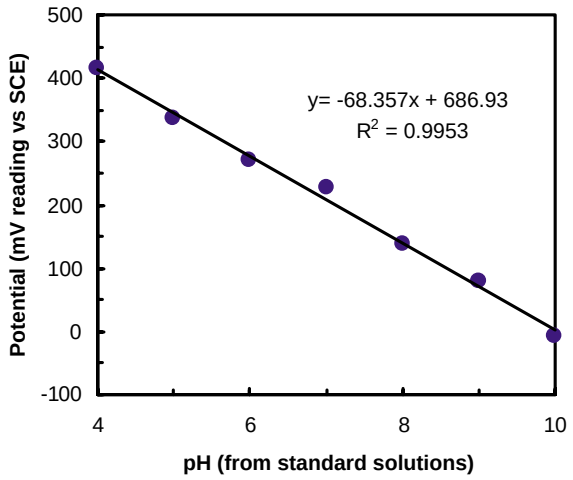

Fig. 3. Potential of the AIROF vs. pH at 25 °C.

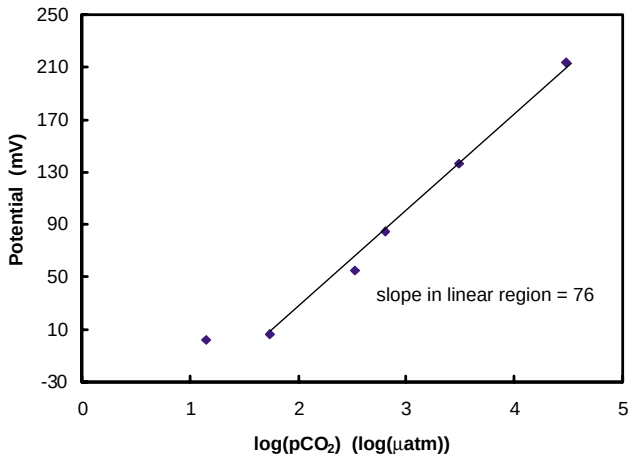

Fig. 4. Calibration curve for CO_2 microelectrode. The slope refers to the linear region only.

somewhat higher than those reported by Zhao and Cai [3], 57 mV per decade, but not as high as those measured by Suzuki et al. [8], about 80 mV per decade.

To accommodate the lower CO_2 concentrations, those out of the linear region in Fig. 4, the data were all fitted to a hyperbolic equation as described by de Beer et al. [4]:

$$E = a + \frac{b \times pCO_2}{c + d \times pCO_2} \quad (12)$$

where E is the electrode potential (mV), and a, b, c, and d are constants which were calculated from non-linear least-square fittings using MS® Excel's solver function. For the data presented in Fig. 4, $a = -53.73$, $b = 4.25$, $c = 0.120$, and $d = -0.011$.

4.3. Response time of the CO_2 microelectrodes

We defined response time as the time required to reach 90% of the final potential [4,8]. Fig. 5 shows a typical response curve of a microelectrode when the partial pressure of CO_2 was changed from 637 to 30,000 μatm, and then from 30,000 to 637 μatm.

The step change in the CO_2 concentration was accomplished by holding the microelectrode in the air (pCO_2 = 637 μatm) and then inserting it into the solution that was equilibrated with 30 matm CO_2. The response time was approximately 2 min when the CO_2 concentration was increased. However, it was four times longer when the CO_2 concentration was decreased (about 8 min). Therefore, to shorten the response time, before the measurements we kept the microelectrodes for at least 20 min at a low CO_2 concentration and then started to measure the CO_2 concentration profile from the top to the bottom of the biofilm (increasing CO_2 concentrations). We selected 3 min as the time needed to measure the CO_2 concentration at a single

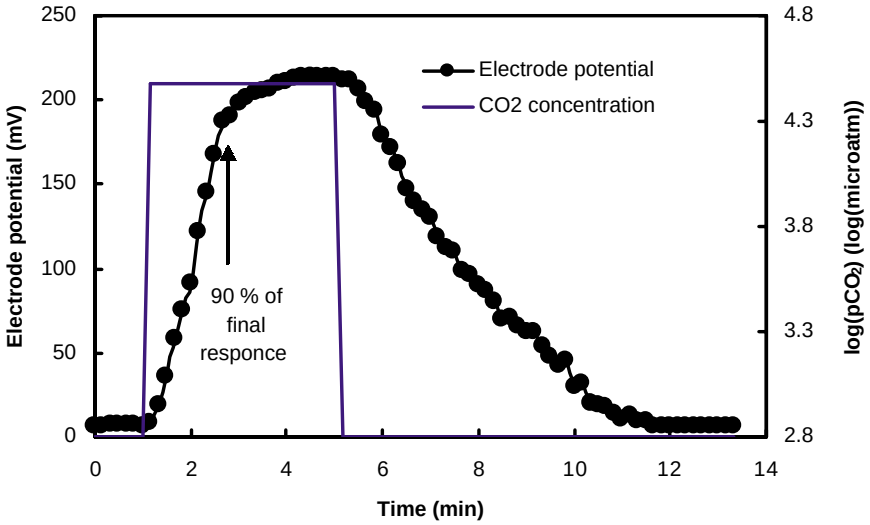

Fig. 5. The response curve of a CO_2 microelectrode. When the CO_2 concentration increased from 637 μatm to 30 matm, the electrode response took 2 min. However, when the CO_2 concentration decreased from 30 matm to 637 μatm its response took 8 min.

location. After we finished measuring a profile, we moved the microelectrode to the top of the biofilm, and waited for at least 20 min to be sure that the electrode was equilibrated at the lowest CO_2 concentration before repeating the measurement.

The important factors affecting the response time of CO_2 microelectrodes have been described in detail by Zhao and Cai [3]; the response time mostly depends on the internal $NaHCO_3$ concentration and the direction of the step change of the CO_2. We used 2 mM $NaHCO_3$, following Zhao and Cai's [3] experimental and theoretical findings. According to these authors, that internal electrolyte concentration responds well to the concentration range of CO_2 we expected to measure (0–10 mg/l). To make sure that the silicone rubber membrane thickness was not limiting the response time, we used only electrodes with membranes thinner than 10 μm. In addition, to make the membrane thinner, we dissolved it partially by immersing the tip of the microelectrode in silicone rubber (50% w/w, dissolved in tetrahydrofuran (THF), (Fluka 87369)/mass THF), following the procedure of Zhao and Cai et al. [3]. This, however, did not improve the response time significantly, indicating that the mass transfer rate through the silicone rubber membrane does not control the response time of the microelectrode, which coincides with the observations described by other authors. It is generally accepted that the rate-limiting factor is the dissociation of H_2CO_3, as explained theoretically by Zhao and Cai [3]. To improve the rate of this reaction, carbonic anhydrase was added to the internal solution, following suggestions of Zhao and Cai [3] and de Beer et al. [4]. Carbonic anhydrases are enzymes that catalyze the conversion of carbon dioxide to bicarbonate, and are important in photosynthesis. They are very active catalysts, with a turnover rate on the order of 10^6 reactions/s.

$$CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+ \quad (13)$$

The response time of our CO_2 microelectrodes is on the order of minutes, which is consistent with other reports [1,3,8,15]. Specifically, Suzuki et al. [8] reported 3 min of response time, which is comparable, although slightly longer than ours. Zhao and Cai [3] reported 2 min as their fastest response time for a microelectrode with a 50 μm tip diameter. Our electrodes show less than 3 min response time, but they have a smaller diameter (20 μm) than those used by Zhao and Cai [3].

While the majority of researchers report several-minute response times for CO_2 electrodes [1,8,15], de Beer et al. [4] reported 10 s! It is difficult to consider those data conclusive because those authors refer to a response time of 10 s in the abstract of their paper but "between 10 s and a few minutes" in the rest of paper. To further confuse the issue of response time, Hanstein et al. [5] reported response times for CO_2 microelectrodes with a few μm tip diameters of between 18 and 63 s. It is possible that the reported discrepancies are caused by the lack of a commonly accepted definition of the response time, and of commonly accepted procedures to measure the response time that would justify comparing the results. To reiterate our observations: our LIX-based CO_2 microelectrodes had a response time conveniently measured in minutes (Fig. 5).

4.4. Life-time of CO_2 microelectrodes

Since AIROF pH microelectrodes are known to have a life-time exceeding several months, we expected that our CO_2 microelectrodes might have a comparable life-time. To test the expected life-time of these electrodes, we stored a microelectrode in the air for 1 month in a clean and dry place, and then compared its calibration curve with that measured when it was freshly made. The sensitivity of the fresh electrode was 74.3 mV per decade ($R^2 = 0.97$), and the sensitivity of the aged microelectrode was 55.6 mV per decade ($R^2 = 0.94$). However, the drift of the aged microelectrode was surprisingly low, only 0.03 mV/h, which is almost 10 times less than the reported drift of the LIX-type CO_2 microelectrodes (0.4 mV/h) [3]. Even though we observe a similar behavior using other microelectrodes, we generally use a new electrode for each experiment because we break them during the last measurements to verify the exact location of the bottom of the biofilm (see the procedure for locating the bottom described previously). The life-time and stability of AIROF microelectrodes were comparable to or better than those of microelectrodes constructed using LIX pH membranes, which are stable for days to weeks [7].

4.5. CO_2 profiles in S. aureus biofilm

The concentration of CO_2 decreased toward the bottom of the biofilm due to CO_2 production. The highest concentration was observed in the middle of the cell cluster (point B, Figs. 6 and 7). However, the CO_2 concentration at point

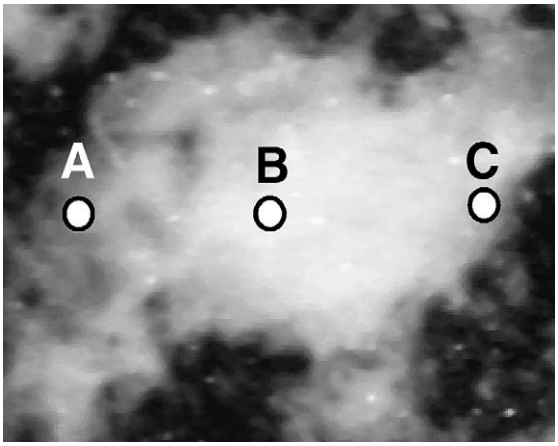

Fig. 6. Light microscopy image of the measurement locations in *Staphylococcus aureus* biofilm. Bright and dark areas represent cell cluster and interstitial void. Points A and C are located near the edge, while B is located in the middle of the cluster.

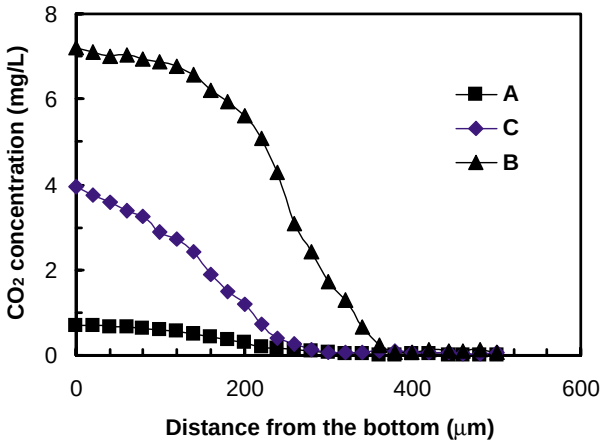

Fig. 7. CO_2 concentration profiles measured at the marked locations in Fig. 6. The thickness of the biofilm was around 400 μm.

C was higher than that at point A. These differences are probably caused by the heterogeneity of the biofilms, uneven distribution of biomass, and uneven biomass density at different locations: CO_2 near the edges of the cell cluster can diffuse toward the interstitial void as well as toward the top of the biofilm.

As expected, CO_2 profiles vary among locations in the microcolony. The steepest profile was found at the center of the microcolony, and less steep profiles were found near the edges. Due to the low drift of the microelectrode signal, the profiles look quite smooth and can easily be used to calculate CO_2 fluxes.

5. Conclusions

1. A Severinghaus-type carbon dioxide microelectrode, with a tip diameter of less than 20 μm, was constructed using AIROF as the internal pH sensor, and used to measure the carbon dioxide concentration profile in a *S. aureus* biofilm.
2. The AIROF pH electrode showed a super-Nernstian response with a slope between 65 and 80 mV/pH.
3. The CO_2 microelectrode showed a higher sensitivity, 65–76 mV per decade (of μatm partial pressure), than the electrodes that use an LIX pH sensing element (57 mV per decade).
4. The CO_2 microelectrode was usable after 1 month of storage and had a lower potential drift (0.03 mV/h) than the electrodes that use an LIX pH sensing element (0.4 mV/h).
5. When tested in *Staphylococcus aureus* biofilms, the CO_2 microelectrode showed a smooth carbon dioxide profile. As expected, the CO_2 partial pressure increased toward the bottom of the biofilm.

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Biographies

Haluk Beyenal received his BSc, MSc and PhD in Chemical Engineering in 1990, 1993 and 1997, respectively, from Hacettepe University, Turkey. After 5 years of post-doctoral research on microsensors and their applications to biofilm processes in Center for Biofilm Engineering Center for Biofilm Engineering, in 2002 he was promoted to research assistant professor in Montana State University. His current research interests are microsensors, microscale chemistry, electrochemistry in biological systems, and biofilm processes.

Catherine C. Davis received her BS with distinction in 1978 from the University of Nebraska-Lincoln and was awarded her MS in Pathology from Washington University-St. Louis in 1980 and her PhD in medical

Microbiology and Immunology from Creighton University in 1990. She has served on the faculties of The University of Iowa and Creighton University and her most recent appointment was as adjunct associate professor at the University of Colorado Health Sciences Center-Department of Oral Biology. Currently, she is Senior Scientist-clinical microbiology for The Procter & Gamble Company, Cincinnati, OH. Her research interest is in the field of the pathogenesis of toxic shock syndrome.

Zbigniew Lewandowski received his MSc in 1969 from the Technical University of Gliwice, Poland, and his PhD in environmental engineering from the Institute of Environmental Engineering, Polish Academy of Sciences, in 1976. He is now professor of Environmental Engineering in the Department of Civil Engineering, Montana State University, and head of Biofilm Structure and Function group at the Center for Biofilm Engineering. His research interests cover various aspects of biotechnology, electrochemistry, and biogeochemistry.