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AN ELECTROCHEMICAL TECHNIQUE TO MEASURE LOCAL FLOW VELOCITY IN BIOFILMS

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Abstract—The limiting current technique and a mobile microelectrode were used to measure local flow velocity in biofilms. The microelectrode was calibrated using the particle tracking technique in conjunction with confocal scanning laser microscopy (CSLM). A relationship between the limiting current density and the local flow velocity was found using nonlinear regression. This relationship was the same whether biofilm was present or not. Therefore, we used the measurements of limiting current density to evaluate flow velocity distribution around biofilm clusters. The flow velocity distribution was affected by the geometry of interstitial voids and by their orientation with respect to the flow direction in the main conduit. Flow velocity measurements using the limiting current technique were faster than similar measurements using particle tracking with confocal scanning laser microscopy. © 1998 Elsevier Science Ltd. All rights reserved

Key words: local flow velocity, local mass transfer, limiting current, confocal scanning microscope, microelectrodes, biofilms.

NOMENCLATURE

A	Active surface area of the microelectrode (m^2)
C_0	Concentration of reacting species in the bulk solution (mol/m^3)
D	Molecular diffusivity of reacting species in the electrolyte (m^2/s)
F	Faraday constant (96,500 C/mol)
I	Limiting current (A or C/s)
i	Limiting current density (A/m^2 or C/s m^2)
n	Number of moles of electrons transferred in the reaction
V	Flow velocity (m/s)
δ_c	Concentration boundary layer thickness (m)

INTRODUCTION

Recent studies have shown that mass transfer rates in heterogeneous biofilms are influenced by convective flow in interstitial voids. Measurements in our laboratory showed that in heterogeneous biofilms oxygen concentration in the voids was significantly higher than in the adjacent cell clusters (De Beer *et al.*, 1994b). This difference in oxygen concentration implies that the voids act as transport channels, thus enhancing mass transport to deeper layers of biofilms. To quantify the intensity of convective mass transport it is necessary to measure flow velocity profiles in the voids.

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Local flow velocity in biofilms was measured by particle tracking in conjunction with confocal scanning laser microscopy (De Beer *et al.*, 1994a; Stoodley *et al.*, 1994) and nuclear magnetic resonance imaging (Lewandowski *et al.*, 1993). Rothemund *et al.* (1993) used laser doppler anemometry but the biofilm interfered with the scattered light and, therefore, the flow velocity was measured only in the bulk liquid outside the biofilm. Confocal scanning laser microscopy and nuclear magnetic resonance require expensive equipment, are time consuming and require highly skilled and highly specialized staff to obtain meaningful results. Therefore, we have developed an alternative technique; microelectrodes sensitive to flow velocity based on the limiting current technique.

The limiting current technique is well known for measuring flow velocity and shear stress near electrode surfaces (Ranz, 1958; Mizushina, 1971; Hanratty and Campbell, 1996). It employs electrodes flush with the wall of a conduit, electroactive species dissolved in a supporting electrolyte and conditions ensuring that the reaction rate at the electrode surface is controlled by the mass transport only (Dawson and Trass, 1972; Selman and Tobias, 1978). Recently, Yang and Lewandowski (1995) used the limiting current technique and a mobile microelectrode to measure distribution of local mass transfer coefficients in biofilms. The tip of their microelectrode formed a microsink consuming electroactive species (ferricyanide, $K_3Fe(CN)_6$)

introduced into a biofilm reactor. The measured limiting current density was a function of the diffusivity of ferricyanide and thickness of the mass transfer boundary layer:

$$i = \frac{I}{A} = \frac{nFC_0D}{\delta_c} \quad (1)$$

When the bulk concentration of the ferricyanide (C_0) was kept constant, the current density, i, was a known function of the mass transfer boundary layer thickness at the tip of the microelectrode, δ_c. The mass transfer boundary layer thickness was controlled by the local flow velocity and remained unknown. Yang and Lewandowski (1995) evaluated the ratio D/δ_c, which they called the local mass transfer coefficient, from the measurements of limiting current density. The adjective "local" implied that the technique measured the mass transfer coefficient at a point near the microelectrode tip rather than the overall mass transfer coefficient to the biofilm.

The goal of this study was to expand the technique described by Yang and Lewandowski (1995) and use it to measure the distribution of local flow velocities in biofilms. We used the same experimental setup as described in the original paper to measure local limiting current density in biofilms. However, we moved one step further and found a function describing the relationship between the limiting current density and the local flow velocity. Using this empirical relationship we calibrated a flow velocity microelectrode and used this electrode to measure local flow velocity profiles in interstitial voids of heterogeneous biofilms.

MATERIALS AND METHODS

Biofilm reactor

The experimental setup is shown in Fig. 1. We used an open channel, flat plate reactor (1), made of polycarbonate, 4 cm deep, 4 cm wide and 75 cm long with a total working volume of 420 ml. The nutrient solution consisted of KH_2PO_4 (0.69 mM), K_2HPO_4 (1.5 mM), $(NH_4)_2SO_4$ (0.079 mM), $MgSO_4{\cdot}7H_2O$ (0.013 mM), glucose (0.040 g/l) was fed to the reactor (12). The hydraulic retention time was less than 4 min to prevent growth of microorganisms in suspension. The solution was aerated in the mixing chamber (7). As inoculum we used frozen stock cultures from the Center for Biofilm Engineering, a mixture of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Klebsiella pneumoniae*. The reactor was operated batchwise for 12 h and then switched to the continuous flow mode. The nutrient solution was recycled through the reactor using peristaltic pumps (Cole-Parmer, Chicago, IL). The biofilm was allowed to grow for 4 to 5 days.

Before measurements, the nutrient solution in the reactor was slowly drained making sure that the biofilm remained intact. The remaining nutrients were washed out with distilled water, then the reactor was carefully filled with the electrolyte solution of 0.025 M $K_3Fe(CN)_6$ in 0.2 M KCl. Fresh electrolyte solutions were prepared before each experiment. We recycled (flow velocity 0.0034 m/s) this solution for 2 h before the measurements

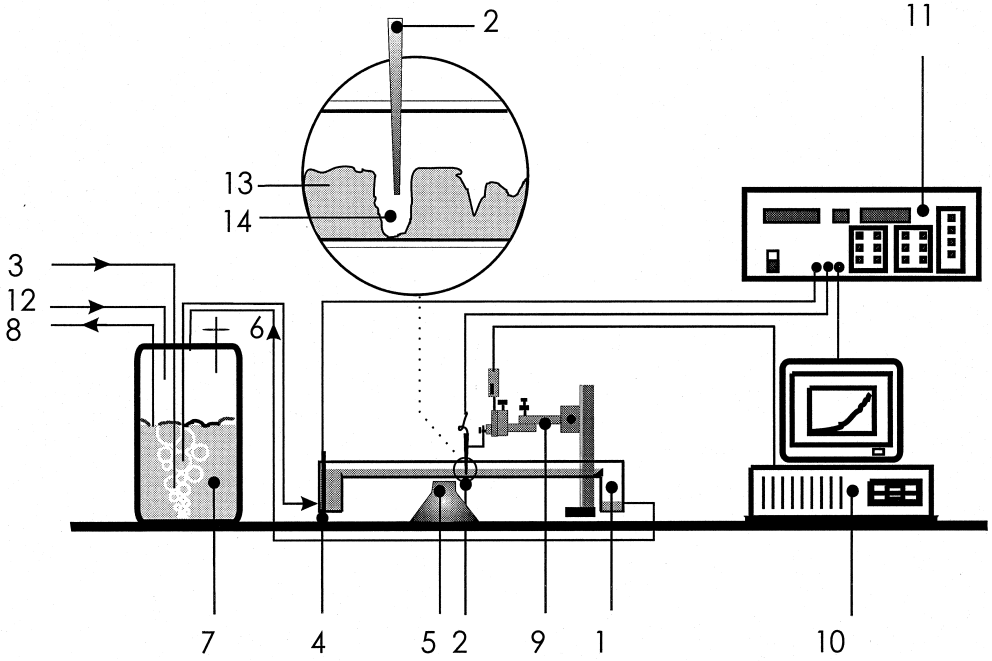

Fig. 1. Experimental setup: (1) open channel, flat plate reactor; (2) microelectrode; (3) air conduit; (4) reference electrode; (5) inverted microscope/confocal scanning laser microscope; (6) air filter; (7) mixing chamber; (8) effluent; (9) micromanipulator; (10) data acquisition system; (11) picoammeter/dc voltage source; (12) fresh feed conduit; (13) biofilm cluster and (14) biofilm void.

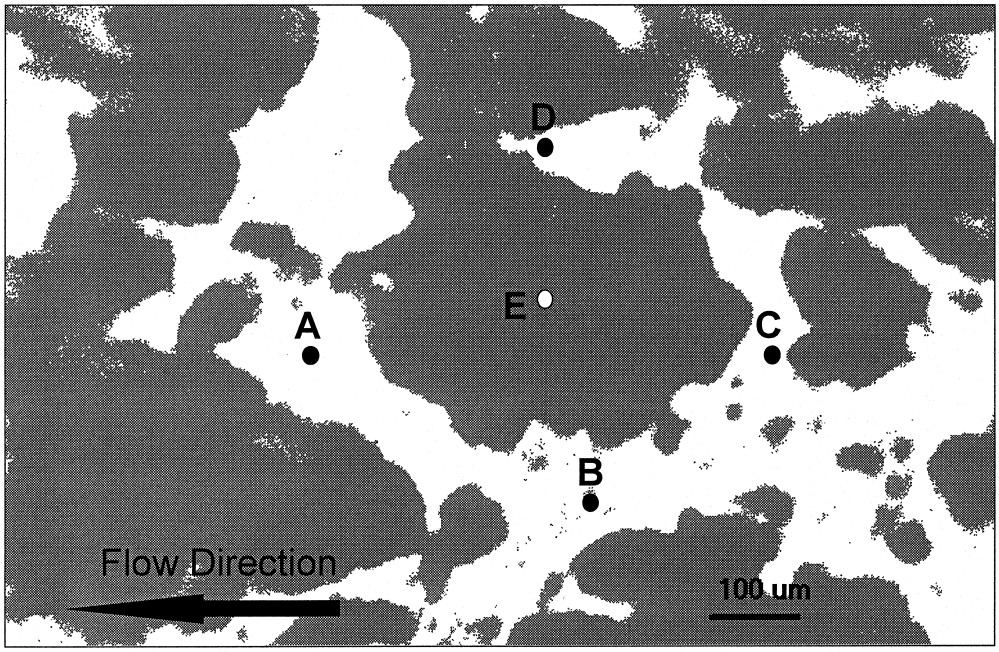

Fig. 2. A microscope image of the biofilm showing where the profiles were measured. White areas represent biofilm voids, dark areas represent cell clusters.

to keep the concentration of ferricyanide in the biofilm uniform.

Microelectrode construction

Microelectrodes were constructed following the procedure described by Yang and Lewandowski (1995). A platinum wire, (California Wire Company, Grover Beach, CA), 100 μm diameter (pure TC grade) was used. The tip of the wire was etched electrochemically (7 V ac) in saturated KCN solution to a diameter 2–6 μm. The wire was rinsed with distilled water and carefully inserted into a capillary made of soda–lime glass. The capillary was positioned in a microelectrode puller (Stoelting Co., Wood Dale, IL), in such a way that the tip of the platinum wire was about 1.5 cm above the heating coil. Heat was slowly increased until the glass around the platinum wire melted and adhered to the wire while the entire capillary dropped down, separated and cooled in the air. The tip of the wire was then exposed by grinding on a rotating diamond wheel (Model EG-4, Narishige Co., Tokyo, Japan), followed by cleaning in a sonication bath with distilled water and acetone. The diameter of the microelectrode tip was measured microscopically and its surface area was calculated. Electrodes with tip diameter less than 10 μm were used.

A voltammetry test was performed for each microelectrode to check its performance. Polarization potential was scanned from –0.2 to –1.2 V in the ferricyanide solution. The microelectrodes having the limiting current in the voltage range between –0.6 to –0.9 V were considered usable. The dependence of the limiting current on the ferricyanide ion concentration was linear, which confirmed that the reaction at the tip of the microelectrode was truly diffusion controlled (Dawson and Trass, 1972).

Microscopic observation, supported by time-lapse photography, revealed that the biofilm structure remained intact for more than 20 h under the continuous recycle of the electrolyte solution. No visible detachment or sloughing was recorded during the measurements. Microelectrode measurements showed that the biofilm was inactive and that neither ferricyanide nor dissolved oxygen were consumed. The results of plate counts showed that the number of viable cells in the biofilm remained relatively constant over first 4 h from the onset of electrolyte recycling.

Measurements of limiting current density

A commercial calomel electrode was used as a counter/reference electrode (Model 13-620-51, Fisher Scientific, Pittsburgh, PA). Hewlett Packard 4140B Multimeter was used as a voltage source and a picoammeter. The microelectrode was polarized cathodically to –0.8 V against the reference electrode that was placed next to it in the reactor.

The microelectrode was mounted on a micromanipulator (Model M3301L, World Precision Instruments, New Haven, CT) equipped with a stepper motor (Model 18503, Oriel, Stratford, CT). Vertical position of the microelectrode was computer controlled using the Oriel, Model 20010, interface.

Location of the electrode tip in the electrolyte solution and in the biofilm was monitored using an inverted microscope (Olympus model CK2, Olympus Optical Co., Japan). The microelectrode was moved from the bulk electrolyte solution toward the bottom of the reactor at preset increments (usually 10 μm). The position of the bottom was set as $z = 0$. The position of the microelectrode, z, was then expressed as the distance from the tip of the microelectrode to the bottom of the reactor.

Custom software was used to control the microelectrode movements and to monitor the limiting current. At each position of the microelectrode, the data acquisition system [Fig. 1(10)] (Model CIO-DAS08PGL, Computer Boards, Mansfield, MA) collected 22 separate readings at a sampling frequency of 1 kHz. The highest and the lowest values were trimmed and the remaining 20 measurements were averaged. The coefficient of variation (CV) was calculated for 20 measurements and compared with the preset acceptable CV. If the measured CV was smaller than that preset, the measurement was accepted, the data stored and the microelectrode moved to the next position. If the calculated CV was higher than that preset, the measurement

was repeated. A relative CV of 5% was used as an arbitrary, acceptable level for all measurements. The profile of limiting current vs depth was displayed on a computer monitor in real time.

Measurements of local flow velocity using particle tracking and confocal scanning laser microscopy

We used the Bio-Rad, model MRC600, confocal scanning laser microscope integrated with the Olympus, model BH2 light microscope, for particle tracking. Detailed procedures of the measurement of local flow velocities in biofilms are given in our previous papers by Stoodley *et al.*, 1994 and De Beer *et al.*, 1994a.

Neutral-density fluorescent latex spheres [density at 20°C, 1,055 kg m^{-3}; excitation wave length (Ex), 580 nm; emission wave length (Em), 605 nm; diameter, 0.216 μm; Molecular Probes, Eugene, OR] were added to the reactor. Velocity profiles in the reactor were obtained by capturing images at different focal depths by raising or lowering the motorized stage. The confocal scanning laser microscope (Ex, 568 nm) was used to capture images by using the Bio-Rad COMOS operating software. Particles traveling across the field of view appeared as dashed lines. The velocity of a particle was calculated from the distance between the dashes and the time taken to scan the number of frame lines crossed by the particle.

Combined measurements of local flow velocity and limiting current density

The measurements of local flow velocity and limiting current density were conducted at specific locations (in X, Y and Z coordinates) in the biofilm. The limiting current density profiles were measured first. Then the microelectrode was removed and the particle tracking technique was used to measure local flow velocity at exactly the same locations as the limiting current density was measured. To evaluate the effect of the biofilm presence on both the local flow velocity profiles and the limiting current density profiles, we measured both parameters in the presence and absence of the biofilm.

Biofilm imaging

To analyze the effects of biomass distribution on the flow velocity we have collected confocal images of the biofilm at several levels. After local flow velocity and limiting current measurements were completed, we replaced the ferricyanide solution with a buffer solution [KH_2PO_4 (0.69 mM), K_2HPO_4 (1.5 mM)] and added, 1% v/v, acridine orange (from Sigma) to stain the biofilm. The solution was recycled for 10 min to complete the staining before the images were taken. Figure 2 shows a confocal image of the studied biofilm taken at the level of the bottom of the reactor. Dark areas represent cell clusters and white areas interstitial voids. Points A, B, C and D were located in the interstitial voids. Point E was located at the top of the cell cluster.

RESULTS

The relationship between the local limiting current density and the local flow velocity measured in a reactor without a biofilm is shown in Fig. 3. The measurements were carried out at three different average flow rates (0.0034, 0.0166 and 0.04 m/s). The following empirical relationship was found between the limiting current density and the local velocity for microelectrodes with tip diameters from 9 to 11 μm:

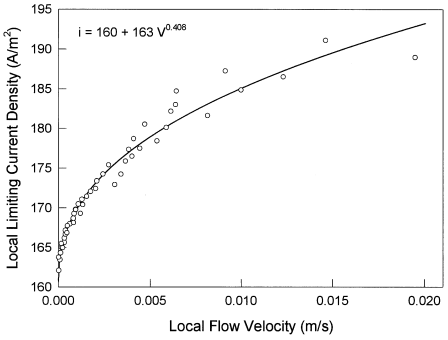

Fig. 3. Relationship between limiting current density and local flow velocity in the reactor without a biofilm (○, data; ———, model).

$$i = 160 + 163V^{0.408} \quad (2)$$

Profiles of both local flow velocity and the limiting current density measured in a biofilm void are shown in Fig. 4. The void was a closed channel 55 μm wide and 150 μm long; average biofilm thickness was 160 μm. The void was parallel to the direction of flow. Average flow rate in the reactor was 0.0166 m/s. At the bottom of the reactor, the flow velocity was zero and the limiting current density had the lowest value, 160 A/m^2. Flow velocity increased with increasing distance from the bottom.

The local limiting current densities measured in the biofilm void (those shown in Fig. 4) and data obtained above the cluster (results not shown) are correlated with the local flow velocities measured at the same locations in Fig. 5. The continuous line in Fig. 5 represents the empirical equation, equation 2, graphically. It shows what the relationship between local flow velocity and local limiting current density would be in the reactor without the biofilm. It is evident from Fig. 5 that the relationship between local flow velocity and the limiting current density is nearly the same in the

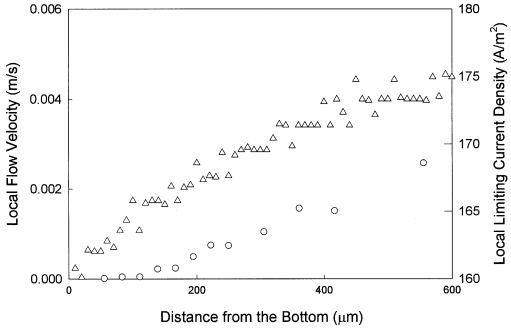

Fig. 4. Local flow velocity and limiting current density depend upon the distance from the bottom of the reactor. The data were collected in a biofilm void (○, local flow velocity; △, local limiting current density).

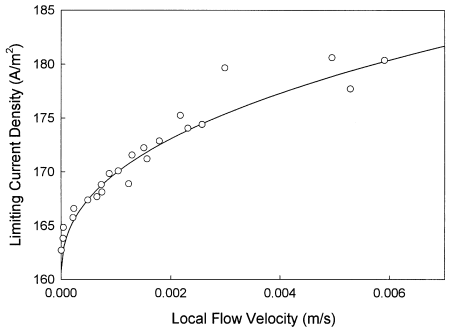

Fig. 5. Relationship between limiting current density and local flow velocity in a biofilm void. The continuous line represents the empirical equation found in Fig. 2, describing the relationship between the limiting current density and local flow velocity in the reactor without a biofilm.

presence and absence of the biofilm. Therefore, an electrode which measures local limiting current density may be used to evaluate local flow velocity.

Following this conclusion we used microelectrodes to measure limiting current density profiles. Then, using equation 2, we evaluated the local flow velocity profiles in a heterogeneous biofilm at locations marked A, B, C, D and E in Fig. 2. The average biofilm thickness at these locations was 140 μm and the average flow velocity in the reactor was 0.0034 m/s. Profiles of limiting current density were measured at each of the marked points in Fig. 2. We evaluated the profiles of local flow velocity at different locations in the biofilm (Fig. 6).

DISCUSSION

Limiting current density is a function of local flow velocity near the tip of the microelectrode. Results in Fig. 5 demonstrate that the relationship between the local flow velocity and the limiting current density at the same location in biofilms is approximately the same in the presence and in the absence of a biofilm. This result not only validates our approach but it also demonstrates that the mass transport to the lower layers of biofilms is much more intensive than we used to think. Based on this, we used the calibration curve measured in the absence of the biofilm (equation 2) to calibrate microelectrodes and to evaluate flow velocity profiles at selected locations in the biofilm.

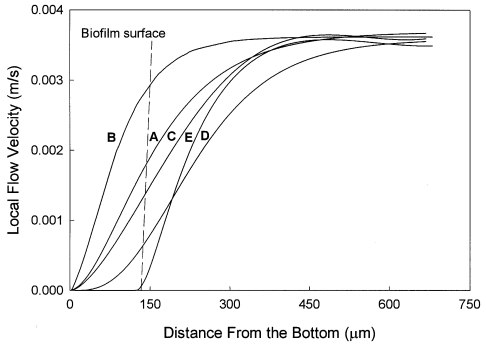

Fig. 6. Local flow velocity profiles measured at positions indicated in Fig. 2.

The results in Fig. 6 show how the biofilm structures affect the flow velocity distribution in biofilm voids. The profiles of flow velocity were evidently different at each location. However, we see regularity in these results showing how the flow velocity profiles in biofilms are affected by the orientation of voids with respect to the direction of flow in the main conduit. The profiles in Fig. 6 show that the highest flow velocity was at point B. This may be a result of point B being located in a channel which was parallel to the flow direction in the main conduit. The flow velocity at point A was lower than that at point B. Point A was located in a channel which was perpendicular to the flow direction in the main conduit. Flow velocity at point C was lower than that at point A. Point C was located near the mouth of a closed channel. Flow velocity at point D was the lowest. Point D was located near the end of a closed channel. The flow velocity profile above and within the cell cluster (point E) shows that the local flow velocity reached zero at the top of the cluster and remained zero within the cluster, which corroborates the notion that the mass transport within cell clusters is mainly controlled by diffusion.

Local flow velocity profiles within the biofilm at each of the five locations marked in Fig. 2 are different. However, they all converge, with different rates, to the same flow velocity, approximately equal to the average flow velocity in the main conduit, 0.0034 m/s.

In our previous works (De Beer *et al.*, 1994a; Stoodley *et al.*, 1994), we measured flow velocity profiles in biofilms using particle tracking and confocal scanning microscopy. In this study, we did similar measurements using limiting current technique and mobile microelectrodes. This latter technique has some limitations; it is insensitive to the direction of flow, can be used only in channels that are wider than the tip of the microelectrode tip, and most likely, gives reliable results only for low flow velocities (at high flow velocities the presence of the electrode may interfere). We found this technique attractive, mostly because it was faster to use than particle tracking with confocal microscopy or the nuclear magnetic resonance imaging. Once the microelectrodes are constructed and calibrated the data can be collected fast and at many locations. Using this technique we demonstrated that local flow velocity profiles in interstitial voids of heterogeneous biofilms depend on the orientation of these voids with respect to the flow direction in the main conduit.

CONCLUSIONS

(1) We have developed and successfully tested an electrochemical technique to measure local flow velocity profiles in heterogeneous biofilms.

(2) This technique gives results comparable to particle tracking with scanning confocal laser microscopy.

(3) Local flow velocities in biofilm voids depend on the void geometry and their orientation with respect to the direction of flow in the main conduit.

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