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Methods in Enzymology

Volume 310

Biofilms

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DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
SCHOOLS OF DENTISTRY AND MEDICINE
UNIVERSITY OF LOUISVILLE
LOUISVILLE, KENTUCKY

1999

ACADEMIC PRESS
San Diego London Boston New York Sydney Tokyo Toronto

METHODS IN ENZYMOLOGY

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forces operating on the surfaces are not known and may be difficult to calculate given the positioning of the rods. Also, biofilm accumulates on all surfaces in the model, unlike flow cell models. This can be a disadvantage when long-term accumulation biofilms may interfere with the operation of electrodes.

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[18] Laminar Flow Chamber for Continuous Monitoring of Biofilm Formation and Succession

By MANFRED S. ZINN, ROBIN D. KIRKEGAARD, ROBERT J. PALMER, JR., and DAVID C. WHITE

Introduction

Microbial biofilms are often found on interfaces between liquids and materials. These biofilms may cause reduction of heat transfer in cooling systems, as well as increased flow resistance and potentiation of microbially influenced corrosion of drinking water systems.[1,2] One means of biofilm control is to alter the chemistry of the substratum to decrease biofilm formation and persistence. Flow cells provide a convenient on-line, nondestructive model for the assessment of biofilm control coating efficacy. This article describes a flow cell that enables quantitative analysis of the biomass under conditions where the flow and chemical properties of the bulk fluid and the substratum composition and topology are controlled. This laminar flow cell system provides valuable comparative data of the efficiency of antifouling (AF) and fouling release (FR) coatings.[3-7] Advantages of the system are (1) laminar flow conditions and controllable defined shear stress over the test surfaces, (2) nondestructive on-line monitoring of the biomass

[1] H. M. Lappin-Scott and J. M. Costerton, *Biofouling* 2, 323 (1993).
[2] J. C. Block, K. Houdidier, J. L. Paquin, J. Miazga, and Y. Levi, *Biofouling* 6, 333 (1993).
[3] A. A. Arrage and D. C. White, *in* "Monitoring Biofilm-Induced Persistence of *Mycobacterium* in Drinking Water Systems Using GFP Fluorescence" (J. W. Hastins, L. J. Kricka, and P. E. Stanley, eds.), p. 383. Wiley, New York, 1997.
[4] A. A. Arrage, N. Vasishtha, D. Sundberg, G. Bausch, H. L. Vincent, and D. C. White, *J. Indust. Microbiol.* 15, 277 (1995).
[5] P. Angell, A. A. Arrage, M. W. Mittelman, and D. C. White, *J. Microbiol. Methods* 18, 317 (1993).
[6] M. W. Mittelman, J. M. H. King, G. S. Sayler, and D. C. White, *J. Microbiol. Methods* 15, 53 (1992).
[7] M. W. Mittelman, J. Packard, A. A. Arrage, S. L. Bean, P. Angell, and D. C. White, *J. Microbiol. Methods* 18, 51 (1993).

0076-6879/99 $30.00

on the test surface, (3) pulsed or continuous inoculation of cells from continuous culture allows testing of antifouling agents in the bulk medium and on the treated test surfaces, and (4) a large test surface enables the harvesting of biofilm cells for quantification using signature biomarker biofilm analyses.

Flow Conditions

A flow is characterized as either laminar or turbulent depending on the strength of its lateral mixing. In laminar flows, excited instabilities are damped, whereas in a turbulent flow they grow to form self-sustained, interacting vortices on many scales. The division between laminar and turbulent flow is characterized for flows in channels (without rotation and thermal effects) by the Reynolds number (Re)[8]:

$$\mathrm{Re} = \frac{\nu d_{\mathrm{H}} \eta_0}{\rho} \tag{1}$$

where ν is a characteristic velocity (cm sec^{-1}), d_{H} is a characteristic length (cm), η_0 is the viscosity in poise (g cm^{-1} sec^{-1}), and ρ is the specific density (g cm^{-3}) of the medium.

For the flow in circular pipes (with d_{H} the diameter of the pipe and ν the center velocity profile) experiments show the onset of turbulent flow at Reynolds numbers of about 1000–2000.[9] The Reynolds number also serves as a basis to compare flows: flow characteristics (e.g., the velocity profile) are identical despite different media, flow velocities, or size of channel as long as the Reynolds numbers are the same.

For flows with more complicated cross sections, the characteristic length is given by the hydraulic diameter:

$$d_{\mathrm{H}} = \frac{4A_{\mathrm{C}}}{s} \tag{2}$$

where A_{c} is the cross-sectional area (cm^2) and s is the perimeter of the same area (cm).

For our experiments, we use a rectangular flow channel with an aspect ratio (width to height) of 75/3. The large aspect ratio results in uniform flow conditions at the center of the channel. Usually, the experiments will be run with Reynolds numbers between 1 and 10. This is well within

[8] B. R. Munson, D. F. Young, and T. H. Okiishi, "Viscous Flow in Pipes." Wiley, New York, 1990.

[9] S. J. Davies and C. M. White, *Proc. Roy. Soc. Lond. A* 119, 92 (1928).

the laminar flow range, which results in a better reproducibility of the experiments and still represents a large range of real flow situations.

The test surfaces (coupons) are located sufficiently far away from the entry region of the channel where the flow develops its profile to equilibrium conditions over a distance of about 30 hydraulic diameters.

Shear Stress

The effect of shear stress on the growth of biofilm was investigated by Peyton and Characklis.[10] They found more rigid and homogeneous biofilms with higher shear stresses. The shear stress τ (dynes cm^{-2}) is highest at the wall and is smaller away from the surface depending on the viscosity (η_0), flow velocity (ν), and distance (d_s) from the surface (with $d_s > 0$ cm):

$$\tau = \frac{3\nu\eta_0}{d_s} \tag{3}$$

Thus, additional information on the strength of adhesion can be gained by increasing the shear stress.

Figure 1 summarizes the characteristic parameters of the flow cell that we have in use (see later).

Verification of Laminar Flow

A well-established method to verify laminar flow conditions is to examine the distribution of a soluble stain that is injected upstream of the flow cell. Several stains can be applied, such as bromphenol blue, blue dextran 2000, food stain, or commercial ink. The flow is documented by photography at time intervals. The typical flow profile of the laminar flow should be observed after at least 30 times the hydraulic diameter. At this point the distribution of the flow velocity over the channel bed remains constant as shown in the photographs as a constant hyperbolic front. The inlet into the flow cell is of great importance. A convex inlet decreases the no-flow region to a minimum (Fig. 2).

Nondestructive and On-Line Monitoring of Biofilms

The quantification of biomass on the substratum surface is a general problem in the study of biofilm growth. This quantification must be on-line and nondestructive for long-term experiments. Fluorescence or biolu-

[10] B. M. Peyton and W. G. Characklis, *Biotechnol. Bioeng.* **41**, 728 (1993).

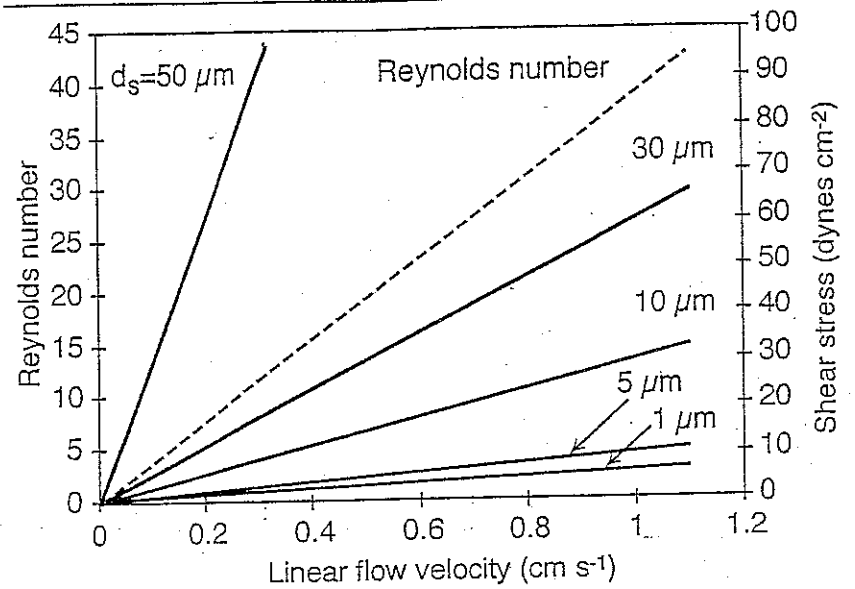

FIG. 1. Flow parameters of the flow channel (hydraulic diameter = 1.52 cm). The linear velocity of the liquid stream determines the fluid dynamics in the flow cell, which can be described by the Reynolds number. The shear stress in the liquid stream increases with the distance (d_s) to the channel surface.

minescence of natural and genetically engineered microorganisms is a nondestructive method to assess AF and FR coating effectiveness. Duysens and Amesz[11] and other authors[12,13] reported that the fluorescence of biomarkers may be used as biomass indicator (see Table I). By this detection method the cells in the biofilm can be followed repeatedly without destruction of the biofilm architecture.

Cleaning of Quartz Windows

For the measurement of fluorescence in the UV range, normal glass windows should not be used. Best results are obtained with quartz windows. Quartz windows need a special cleaning treatment in order to enable reproducible readings (e.g., fingerprints or biofilm formed on the window interfere with tryptophan detection). The procedure consists of several cleaning

[11] L. N. M. Duysens and J. Amesz, *Biochim. Biophys. Acta* 24, 19 (1957).
[12] J.-K. Li and A. E. Humphrey, *Biotechnol. Bioeng.* 37, 1043 (1991).
[13] B. Tartakovsky, M. Sheintuch, J. M. Hilmer, and T. Scheper, *Biotechnol. Progr.* 12, 126 (1996).

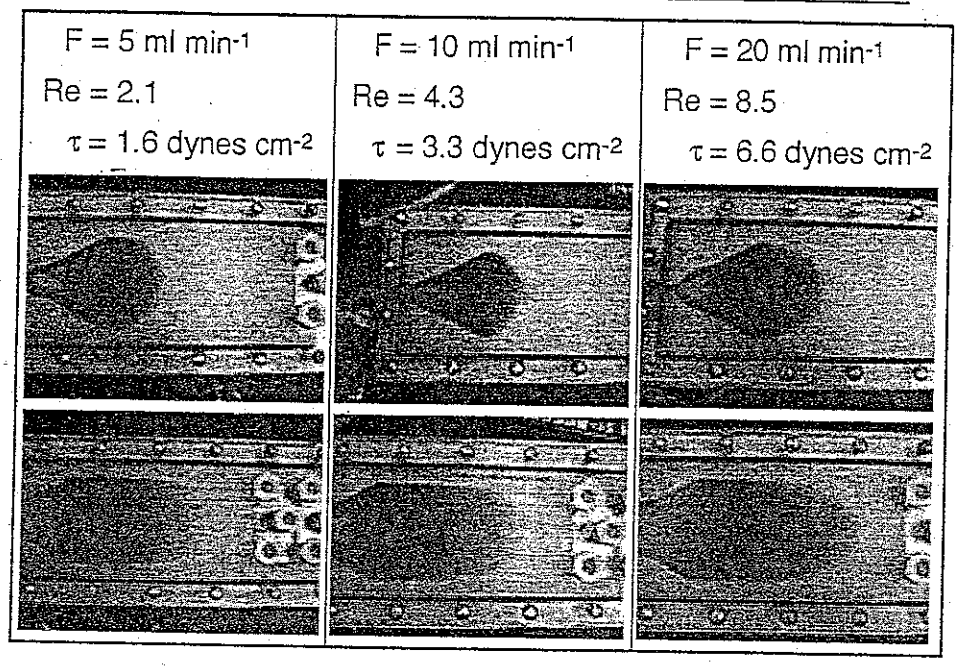

FIG. 2. Verification of laminar flow in a flow cell at three different flow rates. A convex inlet to the flow cell favors the development of the typical laminar flow pattern. After 30 hydraulic diameters, the laminar flow profile is theoretically established and has to remain constant from thereon. (Middle) Inlet of the flow channel, (Bottom) Flow pattern of laminar flow. Figures provided by K. Whitaker.

steps. First, the windows are soaked in a commercial solution of chromic acid/sulfuric acid (Manostat Chromerge, Manostat, New York, NY) overnight, subsequently washed first with distilled water and then with pure methanol. The quartz windows are transferred with forceps into 0.04 *M* KOH in methanol and incubated for about 8 hr. They are washed with this solution as many times as needed until no cloudiness is observed. Finally, the windows are rinsed with distilled water and stored in 65% (w/w) nitric acid until used.

Shortly before use, the windows are washed with distilled water and then methanol. Wearing finger cots, the windows are polished dry with a clean Kimwipe. The windows are then glued with silicone into a hollow polypropylene screw detection port (see later).

Fluorescence and Bioluminescence Measurement

On-line fluorometric measurements are performed with a Fluorolog II spectrofluorometer (Spex Instruments, Edison, NJ) equipped with a

TABLE I
BIOMARKERS TO DESCRIBE PHYSIOLOGY OF BIOFILMS

Biomarker	Detection[a]	Function	Remark	Reference
Tryptophan	Fluorometer (ex 295 nm, em 342 nm)	Biomass	Good correlation to biomass	5
NAD(P)H	Fluorometer (ex 340 nm, em 460 nm)	Cellular activity	Good correlation to biomass during exponential growth	12
ATP	Fluorometer (ex 272 nm, em 380 nm)	Cellular activity		12
Green fluorescent protein	Fluorometer (ex 400 and 480 nm, em 509 nm)	Biomass		3
Lux cassette	Light sensor	Cellular activity and biomass		6

[a] Excitation (ex); emission (em).

bifurcated fiberoptic bundle. Light from a double-grating excitation spectrometer (slit width 2.5 mm) is directed through the fiber-optic cable to the detection window of the flow cell. The emitted light of the biofilm is transported through the same cable to a double-grating emission spectrometer (slit width 2.5 mm). It is important that the spectrometer has a double-grating feature because the reflection of the excitation light can heavily obstruct measurements of emitted fluorescence light.

Bioluminescence of cells containing the lux genes is detected with an Oriel (Stratford, CT) liquid light pipe photomultiplier tube ammeter-monitoring system. All measurements are carried out in a dimmed room in bags made of light-tight fabric.

For long-term studies (>1 day), the detection windows must be cleaned under aseptic conditions with an alcohol pad before each reading.

Description of Flow Cell

The flow cell comprises two separate blocks (Fig. 3) enabling cleaning and efficient sterilization of the flow chamber. The upper block consists of translucent laminated Lexan, whereas the lower block is made of ultrahigh molecular weight polyethylene. The upper block, with a thickness of 2 cm, was designed to be translucent to permit a visual check of the flow cell, e.g., the presence of air bubbles. The chamber, which is cut into the upper

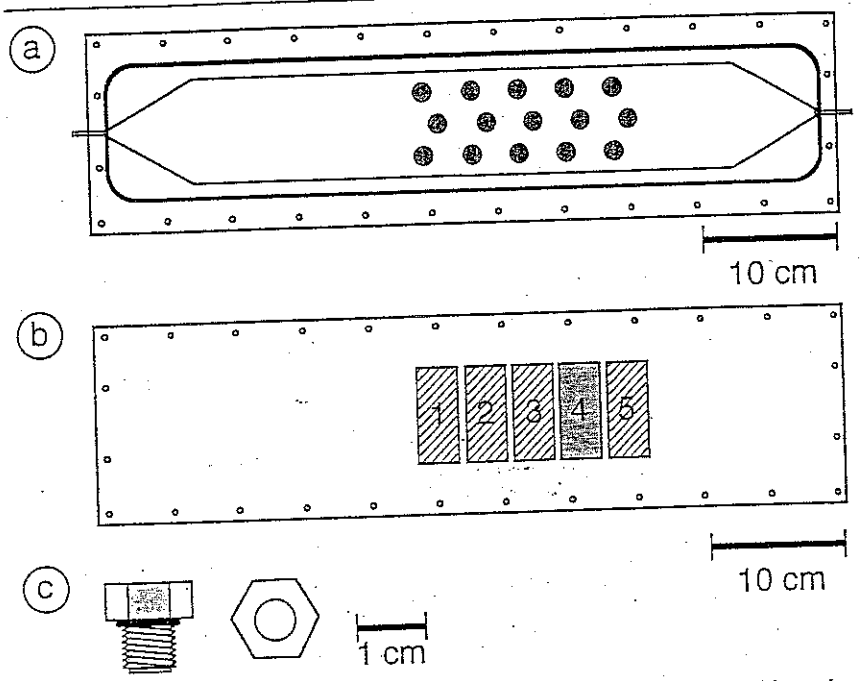

FIG. 3. Schematic view of the flow cell. (a) The upper block of the flow cell is made of translucent Lexan and holds the flow chamber. It also contains the threaded holes with a countersink for the detection windows and the groove for the Viton® O ring that seals the chamber when the flow cell is assembled. (b) The lower block is made of polyethylene and holds five test and reference coupons (numbered in flow direction, number 4 is the test surface). (c) Hollow polypropylene screws are the sample ports for fluorescence and bioluminescence measurements. They are equipped with a quartz window at the lower bottom and a Viton® O ring as a seal.

block, has a depth of 3 mm and holds 75 ml. The chamber is sealed by a Viton® rubber O ring with a cross-cut diameter of 0.5 cm. Metal screws ensure that the O ring is sufficiently compressed to form a seal.

In the bottom half of the channel (1.5 cm thick), five polished stainless-steel coupons (35 × 70 × 3 mm, Metal Samples, Munford, AL) are inserted flush with the flow channel. These coupons are also the carriers for silicone and paint coatings. The hollow polypropylene screws that carry the quartz glass windows (1.2 cm diameter) are placed in the upper half of the flow channel. Thus, fluorescence and bioluminescence measurements can be performed at three locations per coupon.

Sterilization of Flow Cell

Heat sterilization is not possible because the plastic materials warp. The flow cells are assembled with a 3-mm gap between the upper and the lower block and wrapped in a gas-permeable fabric. Sterilization is performed by exposure to ethylene oxide for over 4 hr in a gas chamber of a local hospital. The two blocks are then completely assembled in a laminar flow bench.

Experimental Procedures

The physiology of the bacterial culture used as inoculum should be well defined: the attachment of cells is affected by the growth phase.[14] We generally use a continuous culture at steady state. The dilution rate of the bioreactor is set to about 20% of the maximum growth rate. A continuous sample stream from the bioreactor is then pumped into the flow cell at a rate of 5 ml min^{-1} (Re = 2.1) over a period of 2 hr. Typically, we obtain 10^7 cells cm^{-2} from an inoculum density of 10^6 cells ml^{-1}.

To study bacterial biofouling on test surfaces, sterile medium is provided continuously via peristaltic pumps to the flow cell with sterile medium from a 40-liter reservoir through silicone tubing. The flow is calibrated with a graduated 10-ml pipette inserted into the feed line upstream of the pump through a T connector. The pipette is filled by drawing medium from the reservoir with a 60-ml syringe attached to the pipette through a sterile air filter. Then the tubing to the medium reservoir is clamped while the pump is working; the flow rate is determined by the time required to empty the pipette.

Unfortunately, peristaltic pumps cause pulses in the liquid flow. However, these fluctuations can be damped to some extent by mounting a sterile test tube upside down to the tubing downstream of the pump.

Destructive Biofilm Analysis

At the end of an experiment, a pump is connected to the outlet tubing and the flow cell is tilted 45° with the inlet up. The feed tubing is removed and the pump is started to drain the flow chamber at a rate of approximately 100 ml min^{-1}. The upper block is immediately unscrewed and the test coupons are collected in sterile petri dishes containing wet paper towels to reduce desiccation of the biofilm. The cells are quantitatively removed from the coupons by probe sonication (three times for 1 sec at 20 W) in

[14] S. McEldowney and M. Fletcher, *J. Gen. Microbiol.* 132, 513 (1986).

10 m*M* phosphate buffer (pH 7.0) using 1.131-cm^2 glass O-ring extractors (Kontes Glass, Vineland, NJ). The cells can then be processed further for acridine orange direct counts,[15] lipid extractions,[4] or protein analysis.[16]

Data Analysis

From each coupon three data points are collected and are analyzed statistically (*t* test at a 95% probability level). Generally, three flow cells are run at the same time in order to improve the statistical analysis. For long-term experiments, data of an untreated metal coupon in position 3 (Fig. 3) are taken as a benchmark.[4]

[15] G. A. McFeters, A. Singh, S. Byun, P. E. Callis, and S. Williams, *J. Microbiol. Methods* **13**, 87 (1991).
[16] M. W. Mittelman, D. E. Nivens, C. Low, and D. C. White, *Microb. Ecol.* **19**, 269 (1990).