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Immobilized Cells: Basics and Applications

Local Mass Transfer Coefficients in Bacterial Biofilms Using Fluorescence Recovery After Photobleaching (FRAP)

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Abstract

Pure culture *Pseudomonas putida* biofilms were cultivated under controlled conditions to desired overall biofilm thicknesses. Said biofilms were placed within half-cell diffusion chambers to estimate, from transient solute concentrations in each chamber, the effective diffusion coefficient for several macromolecules of increasing molecular weight and molecular complexity. Results of the half-cell studies were found to be erroneous due to the existence of microscopic water channels or crevasses that perforate the polysaccharidic gel matrix of the biofilm, sometimes completely to the supporting substratum. Thus, half-cell devices measure an average transfer coefficient that overestimates the true, local flux of solutes in the biofilm alginate gel matrix.

An alternative analytical technique was refined to determine the local diffusion coefficients on a micro-scale in order to avoid the errors created by the water channels. This technique is based upon the Fluorescence Return After Photobleaching (FRAP) which allows image analysis observation of the transport of fluorescently labeled molecules as they migrate into a micro-scale photobleached zone. The technique allowed us to map the local diffusion coefficients of various solute molecules at different horizontal planes and depths in a biofilm. These maps also indirectly indicate the distribution of water channels in the biofilm. FRAP results illustrate a significant reduction in macromolecule transport coefficients in biofilm polymer gel versus the same value in water, with the reduction being dependent on solute molecule size and shape.

Determination of Diffusion Coefficients

Half-Cell Diffusion Chambers

Translational diffusion coefficients for a solute in a through a permeable material can be determined from an unsteady-state experiment where two well-mixed chambers (termed "half-cells") are separated by a known thickness of the permeable material. At time equal zero, the diffusing solute in question is added to the larger half-cell in a sufficient amount to maintain a constant concentration. Concentrations of solute in both half-

cells are then determined as a function of time with diffusion coefficients calculated from Eqn. (1),

$$ln\left[\frac{C_I(t)-C_{II}(t)}{C_I(t=t_1)-C_{II}(t=t_1)}\right]=\frac{A}{K}\left(\frac{1}{V_I}+\frac{1}{V_{II}}\right)(t-t_1) \quad (1)$$

where V_I = volume of cell I (L^3); V_{II} = volume of cell II (L^3);
C_I (t) = concentration of solute at time t in cell I, (M L^{-3})
C_{II} (t) = concentration of solute at time t in cell II, (M L^{-3});
$C_{I,\ II}$ (t @ t_1) = concentration of solute in cell I or II, respectively, at the onset of pseudo-steady state, time t = t_1, (M L^{-3});
t = time (t); A = surface area connecting the two half cells, (L^2); and
K = the overall composite mass transfer coeffficient, (L t^{-1}).

K can be defined for this work by Eqn. (2),

$$K^{-1}=\left[\frac{L_M}{\mathcal{D}_{eff\text{-}m}}+\frac{L_{biofilm}}{\mathcal{D}_{eff\text{-}biofilm}}\right] \quad (2)$$

where L_M = thickness of the permeable membrane separating the two half cells and employed as a support for biofilm accumulation, (L);
$L_{biofilm}$ = thickness of the biofilm, (L);
$\mathcal{D}_{eff\text{-}m}$ = diffusivity of solute through support membrane alone, (L^2 t^{-1}); and
$\mathcal{D}_{eff\text{-}biofilm}$ = diffusivity of solute through biofilm, (L^2 t^{-1}).

Half-cell chambers allow estimations of Fickian diffusion coefficients as the result of a concentration gradient, averaged over the entire structure of the biofilm. The design and dimensions of the half-cell device employed in these studies is detailed in Drummond **[1]**. Two rectangular chambers or half-cells, one with a 147 mL working volume the other with a 475 mL working volume, were bolted to either side of a separating plate assembly. One version of the separating plate was completely solid for half cell leak tests and mixing studies; a second version contained a 15.9 cm^2 circular opening covered by a 0.1 μm pore size Costar Nuclepore™ polycarbonate membrane for determination of solute transport rates through the membrane only; and, for the actual biofilm solute transport rate tests, a the second plate assembly consisted of a composite membrane-biofilm layer.

Fluorescent Return After Photobleaching (FRAP) Studies

FRAP is in concept a simple technique **[2]**. A small region of a surface or volume containing mobile fluorescent molecules (or fluorescently labeled macromolecules) is exposed to a brief intense pulse of light thereby causing irreversible (assumed but often times not true) photochemical bleaching of the fluorophore in that region. Transport properties of the

recovery which occurs due to transport of unbleached molecules migrating into the bleached area from the unirradiated surroundings. Overall fluorescence is achieved using a separate source of light with a much attenuated intensity. FRAP, using unpolarized laser light, provides direct estimation of *tracer* diffusion coefficients; a diffusion parameter that is not generated due to a concentration gradient as in half-cell studies. Direct FRAP can provide estimates of local tracer diffusion coefficients in liquids or gels since the initial bleaching beam can be focused to photobleach a region of very small dimensions. Thus, numerous FRAP tests can be carried out over a large section of sample.

Theory used in FRAP analysis was developed by Axelrod *et al.* **[2]** for the idealized case of pure 2-D diffusion monitored by a laser beam with a Gaussian intensity profile. The normalized recovery curve is given by Eqn. (3),

$$f(t) = \sum_{n=0}^{\infty} \frac{-\kappa^n}{n!} \frac{1}{1 + n[1 + (\frac{2t}{\tau_d})]} \tag{3}$$

where τ_d = the 2-D characteristic diffusion time defined by

$$\tau_d = \omega^2/4D \tag{4}$$

where ω is defined as half the width of the Gaussian profile of the focused laser spot usually determined at e^{-2} times the height of the profile. D (cm^2/sec) is defined as the *tracer* diffusion coefficient in the biofilm.

In Eqn. (3), κ is a bleach constant that depends on the sensitivity of the system for bleaching and is experimentally related to the percenatge bleached through Eqn. (5),

$$\% \text{ Bleach} = 100\%[(\kappa - 1 + e^{-\kappa})/\kappa] \tag{5}$$

The fluorescence recovery is given by Eqn. 6,

$$F(t) = F(i)\ \{\ 1 - R[1 - f(t)]\} \tag{6}$$

where F(i) = the intensity of the bleach spot before bleaching and R is the mobile fraction of solute defined by,

$$R = [F(\infty) - F(0)]\ /\ \{F(i) - F(0)\} \tag{7}$$

where $F(\infty)$ = the normalized intensity of the bleached spot at an "infinite time" after bleaching and F(0) = the normalized intensity at t = 0 after bleaching.

The FRAP apparatus was developed around a Reichert-Jung UV/VIS microscope and digital image video monitor system (Figure 1). The microscope stage was under the control of a computerized microstepping

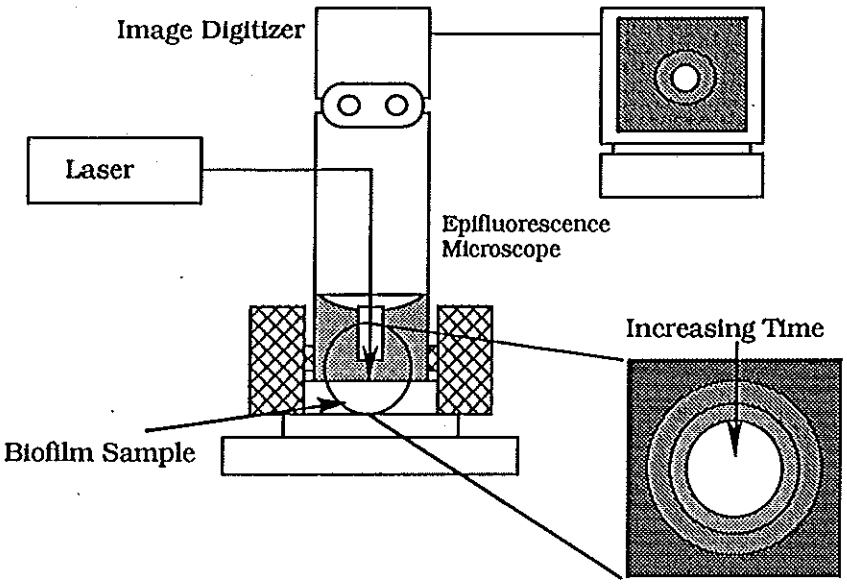


Figure 1. Schematic of microscopic fluorescence return after photobleaching system.

motor which allowed programmed photobleach-then-recovery observations at various points in the biofilm across a horizontal plane at any depth. The increment of stage adjustment could be as small as 5 μm. Translation of the stage insured that no point in the biofilm is exposed to bleaching light more than once. Biofilms were cultivated upon glass support surfaces rather than the polycarbonate membranes required for the half-cell diffusion studies. The sample was covered in buffer nutrient solution minus electron donor, to prevent the sample from drying out. To fluoresce the entire sample, the low intensity UV light source and filters of the epifluorescent microscope, were used; less than 1% bleaching of the fluorophore by the microscope light was observed. For all biofilm experiments, a 1 sec bleaching burst was employed while the fluorescence return was recorded over a period of several minutes or until florescence in the burst zone returned to pre-burst levels. Light intensity was adjusted to achieve produce a 25-40% bleach in the burst zone. The bleach beam was produced by a 15-W Coherent argon ion laser run at 2-W output at the 514.5-nm line. Note that significant light is lost as the beam travels from the laser, through the microscope and to the sample; approximately 100 mW of power actually strikes the biofilm.

Results

Half-cell Diffusion Studies

At least three diffusion trials for each solute were carried out for each biofilm thickness. Diffusivity estimates were based on calculations using

Eqns. (1) and (2) and data considered valid during the steady-state portion of each diffusion trial (*i.e.*, after a time period t_1 which varied with solute and biofilm thickness) (Figure 2). Results of the effective diffusivity calculations based on Eqns. (1) and (2) are summarized in Table 1. The immunoglobulin, IgG, proved to adsorb tenaciously to both diffusion chamber walls and biofilm polysaccharide matrix to such an extent that a true diffusivity could not be determined. Also reported in Table 1 is the ratio of the estimated solute diffusivity in biofilm relative the calculated value for the solute in pure water.

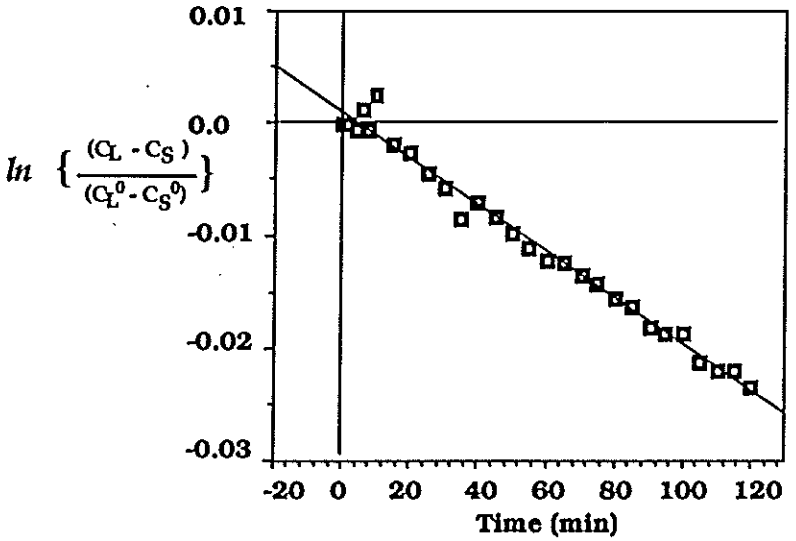


Figure 2. Half cell diffusion data processed as per Eqn. 1. Solute here is fluorescein at 30 oC.

Based on these half-cell studies, overall diffusivities for the various solutes did not differ significantly from their value in water nor from each other. These results insinuate very little influence, if any, of *(a)* the biofilm on the transport rate from one half-cell to the other and *(b)* of the size or shape of the solute molecule on the transport rate through the biofilm. These observations were neither expected nor accorded much credibility.

Localized FRAP Diffusion Studies

Figure 3 illustrates a FRAP recovery curve, normalized to the pre-bleach fluorescence, following a 1 sec photobleach. Estimates of the local diffusion coefficient are made by correlation between the theoretical fluorescence recovery curve and experimental observation, with the only

TABLE 1. Diffusion coefficients for fluorescently labeled molecules in a bacterial biofilm of 180 μm using diffusion half-cells.

Solute, *i*	Molecular Weight	Diffusion Coefficients (cm^2/sec) x 10^{+7} In Pure Water*	In Biofilm	$\mathcal{D}_{biofilm}$ / $\mathcal{D}_{i\text{-}water}$
FITC§	332.	55.0	54.0	0.91
Dextran	10,000.	2.2	1.96	0.89
Dextran	70,000.	1.7	1.44	0.85
BSA§	68,000.	6.9	5.9	0.86
IgG§	146,000.	3.8	NA	NA
HK§	102,000.	5.9	4.1	0.69
Catalase	225,000.	4.1	2.78	0.67
DNA	$3.2x10^6$	0.008	0.005	0.63

abbreviation definitions after Table 3.

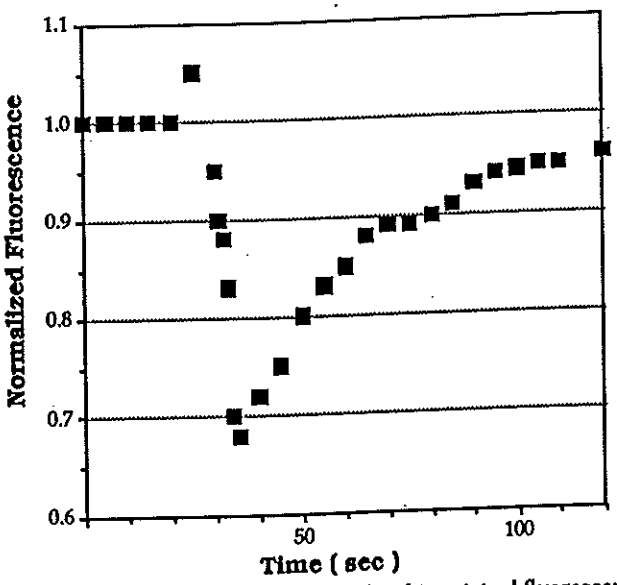


Figure 3. Example of FRAP test , normalized to original fluorescence prior to bleach. Data for FITC - labeled dextran (MW = 10' 000). Photo-bleached zone was a circle of diameter 5 μm in radius.

adjustable parameter being the diffusivity. As the FRAP tests are repeated across a plane in the biofilm, areas of locally specific d_{solute} can be determined for all solutes. As depth is changed and another horizontal scan is carried out, it became evident within the water channels that $D_{solute} \cong D_{water}$. A summary of the various solute (D_{solute} / D_{water}) taken within biofilm polymer-cell clusters is provided in Table 2. Figure 4 provides a summary for the solute dextran (MW = 10, 000) of several horizontal FRAP scans as a function of depth in the biofilm. Figure 4 illustrates regions where mass transfer coefficients are approximately equal to those of the solute in water while in other locals the transport rates of the solute are greatly reduced.

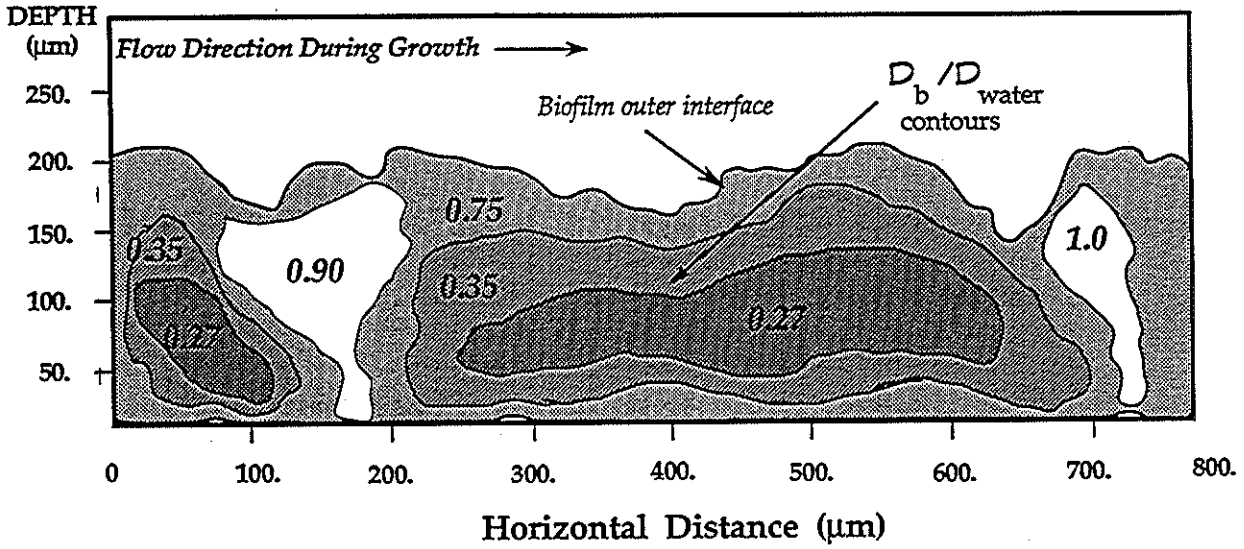
TABLE 2. Diffusion coefficients for fluorescently labeled molecules in water and in a bacterial biofilm using the FRAP method. Biofilm was 232 μm in overall thickness; results here are averages from FRAP scans taken within the same horizontal plane at an depth of 120 μm.

Solute *i*	Molecular Weight	Diffusion Coefficients [(cm^2/sec) x 10^{+7}] In Pure Water*	Biofilm Location: Water Channel	Biofilm Location: Cell Cluster	$D^{\star}_{i\text{-}biofilm} / D_{i\text{-}water}$
FITC§	332.	55.0	54.0	50.	0.91
Dextran	10,000.	2.2	2.0	0.62	0.28
Dextran	70,000.	1.7	1.7	0.26	0.15
BSA§	68,000.	6.9	6.8	3.73	0.54
IgG§	146,000.	3.8	NA	NA	NA
HK	102,000.	5.9	5.0	2.89	0.49
Catalase	225,000.	4.1	4.0	1.27	0.31
DNA	3.2×10^6	0.008	0.007	0.0015	0.19

* calculated from Einstein's Equation: $d_{i\text{-water}} = k_b T / 6 \pi \mu r_s$
where k_b = Boltzman's constant; T = temperature, °K; μ = viscosity of solvent:solute mixture at temperature, T; and r_s = Stokes molecular radius.

§ FITC = Fluorescein; BSA = bovine serum albumen; IgG = G class of human immunoglobulins, antibodies; HK = hexokinase.
NA = not applicable; results for IgG suspect due to binding of the immunoglobulin to biofilm matrix and cell wall.
☆Ratio reported are for values taken in biofilm cell clusters relative to pure water values.

Figure 4. Spatial Differences in Diffusion Coefficients of Dextran (MW = 10' 000) in a Pure Culture Biofilm as Determined by FRAP. Values Reported as Fractions of Dextran Diffusivity in Pure Water Solution at 30 C.



Conclusions

- Mass transfer properties (or any physical, compositional, rheological property) of a biofilm can no longer be considered as unique properties of an entire biofilm but are rather spatially dependent on biofilm structure.

- Results show that local mass transport coefficients can vary dramatically over very small distances, both in biofilm depth (perpendicular to the outer interface) and across any plane parallel to the substratum.

- Mass transfer coefficients of macromolecules in biofilm clusters as estimated by the FRAP technique decreased with molecular weight within a homologous chemical series.

- However, the decrease in diffusivity for linear molecules such as dextrans and the linear cDNA fragment, with increasing molecular weight was more severe than observed for similar molecular weight changes in globular proteins.

- The decrease in dextran (MW = 68' 000) diffusivity in biofilm versus the decrease in BSA (70' 000) diffusivity (molecules of similar molecular weight) were not comparible. The greater decrease in dextran diffusivity versus that of the protein was attributed the solute molecular shape (molecule length) relative to the gel matrix configuration.

References

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2. Axelrod, A., Koppel, D. E., Schlessinger, J., Elsen, E., Webb, W. W. Biophys. J. 1976; 16: 1055-1069.

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