Determination of Diffusion Coefficients in Biofilms by Confocal Laser Microscopy

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Microbial exopolymer may hinder the diffusion of nutrients, antibiotics, and other materials to the cell surface. Studies of diffusion in biofilms have been limited to indirect measurements. This study demonstrated the use of fluorescein and size-fractionated fluor-conjugated dextrans in conjunction with scanning confocal laser microscopy to directly monitor and determine diffusion coefficients within biofilms. The monitoring approaches were simple and, when combined with computerized image collection, allowed assembly of a data set suitable for calculation of one-dimensional diffusion coefficients for biofilm regions. With these techniques, it was shown that regional variability in the mobility of the dextrans occurred within mixed-species biofilms. Some regions exhibited rapid diffusion of all test molecules, while adjacent regions were only penetrated by the lower-molecular-weight compounds. The effective diffusion coefficients (D_e) determined in a mixed-species biofilm were a function of the molecular radius of the probe (i.e., fluorescein, D_e = 7.7 × 10^{-8} cm^2 s^{-1}; 4,000 molecular weight, D_e = 3.1 × 10^{-8} cm^2 s^{-1}; and 2,000,000 molecular weight, D_e = 0.7 × 10^{-8} cm^2 s^{-1}). These results demonstrated that diffusion in the biofilm was hindered relative to diffusion in the bulk solution. The study indicated that in situ monitoring by scanning laser microscopy is a useful approach for determining the mobility of fluorescently labeled molecules in biofilms, allowing image acquisition, appropriate scales of study, both xy and xz monitoring, and calculation of D_e values.

The exopolysaccharide matrix of microbial biofilms may act to various degrees as a diffusion barrier, molecular sieve, and adsorbent. These properties of the biofilm matrix have frequently been invoked to account for altered metabolism, increased resistance to antimicrobial agents, development of anaerobic microzones, structural heterogeneity in biofilms, and protection from predation and desiccation (10, 12, 14, 26, 32). Bacteria live in a diffusion-dominated environment and, as solitary organisms, are unable to alter the environment in which they live (17). The development of an extensive extracellular matrix and growth in attached communities may be important for the maintenance of optimum environmental conditions. Thus, measuring the mobilities of various chemical species within the biofilm matrix is important for understanding dynamic phenomena within biofilms and the function of extracellular polymers in these communities.

Traditional approaches to the measurement of diffusion include the use of dialysis tubing, membranes, and agar gels as model systems, with extrapolation of the results obtained to microbial systems. Measurements have also been obtained by using particulate microbial biomass filtered onto a membrane (1, 25, 27). Diffusion coefficients may be determined with microelectrode techniques and other methods whereby the distribution of the compound of interest can be determined, its rate of consumption or change can be known, and the transport coefficients can be calculated (5, 11, 31). However, these indirect approaches are not simple, do not allow in situ visualization, do not provide information about the spatial variability of diffusion processes, and are not at an appropriate scale for biofilm studies.

The structural matrix of microcolonies and biofilms can be probed with size-fractionated fluorophore-conjugated dextrans, Ficolls, or other inert tracer particles in conjunction with scanning confocal laser microscopy (SCLM). This technique has also been used for in situ monitoring to examine penetration and distribution of defined probes within biofilms and bioaggregates, demonstrating the existence of channeling and sieving in microbial biofilms (6, 7, 22). Techniques such as fluorescence recovery after photobleaching (FRAP) (2, 3, 23, 28) have also been suggested for the measurement of diffusion and mobility of defined fluorescently labeled materials within natural systems such as biofilms (22). The rationale for this approach is that the motion of macromolecules will provide information on various characteristics of their environment. Diffusion coefficients may then be determined by appropriate calculations (2, 3). Thus, in situ monitoring and FRAP with confocal laser microscopy may be used to study diffusion in microcolonies or biofilms and to examine the spatial and temporal variation of these properties. Procedures such as FRAP have been applied extensively in biophysical studies (2, 3, 23, 28) but not in microbiological systems.

This study evaluated the efficacy of FRAP and an alternative nondestructive SCLM monitoring procedure for visualization and quantification of the mobilities of a panel of fluor-conjugated dextrans in the microenvironments surrounding undisturbed microcolonies and in mixed-species biofilms. In addition, a simplified approach for the calculation of effective diffusion coefficients in biofilms is presented.

MATERIALS AND METHODS

Bacteria, continuous-flow slide culture, and media. These studies were carried out with a Pseudomonas fluorescens strain and a mixed-species microbial consortium. The pseudomonad was cultivated in continuous-flow slide culture with 10% strength Trypticase soy broth as the growth medium. The growth characteristics of this strain have been described pre-
viously (8, 19, 21). The mixed microbial consortium was isolated from soil and maintained in a 250-ml continuous culture system (dilution rate, 0.5 h⁻¹) with the herbicide diclofop methyl as the sole carbon and energy source (35). Diclofop methyl-degrading biofilms were grown and examined in multichannel flow cells (1 by 3 by 50 mm channels) irrigated with a mineral salt medium supplemented with 14 mg of diclofop methyl per liter. The multichannel flow cells have been described in detail by Wolfardt et al. (34). All flow cell channels were inoculated with 0.1 ml of culture from an overnight batch culture in the case of P. fluorescens or the continuous culture system for the microbial consortium. The bulk laminar flow rate in the multichannel flow cells was maintained at 0.2 mm s⁻¹ during development of the consortium biofilms and 1 mm s⁻¹ during P. fluorescens experiments. The biofilm development period prior to diffusion studies was 24 h for P. fluorescens and >21 days for the biofilm consortium. These time periods have been shown previously to allow the biofilms to achieve nearly steady-state conditions (18, 34). Both the P. fluorescens and consortium biofilms have been studied extensively and represent well-defined systems (18, 34).

SCLM, in situ monitoring, and image analyses. In situ monitoring was performed with the Bio-Rad MRC 600 SCLM equipped with an argon laser and standard filter blocks. The SCLM system was controlled with a Northgate 486 host computer and the software provided by Bio-Rad. The laser system was mounted on a Nikon SA photomicroscope equipped with 100× and 60× (1.4 NA) objective lenses. The microscope stage was also computer controlled for xy and xz positioning. Fluorescein isothiocyanate (FITC)-conjugated dextrans (Molecular Probes, Eugene, Oreg.) were introduced into the flow cell (0.2 ml at 0.1 ml s⁻¹), displacing the contents of the bulk phase, at time zero. At the same time, a subroutine (Fig. 1) was activated which instructed the SCLM to collect single images at the same location (5 μm from the glass surface) at 5-s intervals. This process was repeated at five replicate locations within the flow cell for each of the test compounds: fluorescein and dextrans with molecular weights of 4,000, 40,000, 70,000, 500,000, and 2,000,000 (4K, 40K, 70K, 500K, and 2,000K fluor-conjugated dextrans). Although an \( F_v/F_n \) ratio (relative values of fluorescence intensity, where \( F_v \) is the grey value at any time and \( F_n \) is the maximum grey value) is used for determination of the effective diffusion coefficient, \( D_v \), probe solutions were prepared to provide comparable fluorescence yield, i.e., number of FITC molecules per mole of dextran. The automatic gain and black-level controls of the MRC 600 were turned off during these experiments and set manually. Monitoring was done with a 1% transmission setting for laser intensity. This laser setting has previously been shown to produce minimal artifacts during studies of attached bacteria (7) and for obtaining recovery images in FRAP (3). The result of each monitoring experiment consisted of a file (30 images or 150 s) representing the diffusion of the test compound into the biofilm. An additional series of 30 images recording the diffusion of the probe out of the biofilm were collected after the bulk phase of the flow cell was displaced with fluor-free medium. These images were then processed to examine the change in average grey level within a region or regions of each image series over time.

**Photobleaching experiments.** Photobleaching and recovery monitoring were carried out with the 488-nm line of the Bio-Rad MRC 600 laser system. The software controlling the system existed as a command file in the SOM software package. The bleach pattern used was either a rectangular box or a single scan line. Box scanning was carried out with the zoom function (zoom 5) to create a bleached rectangle; the recovery of fluorescence was then monitored with the lowest-intensity monitoring beam at zoom 1 (Fig. 2). After \( xz \) bleaching of a single line, \( xy \) recovery images (512 by 768 pixels) were obtained at 5-s intervals at the lowest-intensity laser setting as a monitoring beam. The area of the bleached region was small (<1% of the total biofilm area), and the fraction of total fluorescence bleached was on the order of 50%, to avoid artifacts and dilution of the fluorescence signal. A minimum of five replicate determinations were made at randomly selected

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**FIG. 1.** Image acquisition file code for the Bio-Rad MRC 600.

**FIG. 2.** SCLM photomicrographs showing (A) bleach pattern, (B) prebleach fluorescence level, (C) bleached area at time zero, (D) recovery image after 60 s, (E) recovery image after 180 s, and (F) recovery image after 240 s.
locations within the biofilm. This is one of the simplest forms of photobleaching (3, 16).

**Calculation of diffusion coefficients.** Within an aqueous solution, the diffusion of molecules on the order of those investigated is adequately described by Einstein’s solution, $D_{\text{m}} = kT/F_{\text{m}}$, where $D_{\text{m}}$ is the bulk solution diffusion coefficient, $k$ is Boltzmann’s constant, $T$ is temperature (degrees Kelvin), and $F_{\text{m}}$ is the molecular friction coefficient in bulk solution. Assuming that the diffusing molecule is spherical, $F_{\text{m}}$ may be determined by using the formula $F_{\text{m}} = 6\pi\eta r_s$, where $r_s$ is the solute radius and $\eta$ is the dynamic viscosity of the solvent. These relationships were used to estimate the diffusion coefficient for the probes in aqueous solution ($D_{\text{aq}}$) at 22°C (Table 1).

Grey-level values for each time period in FRAP or monitoring experiments were then plotted as $F/F_{\text{p}}$, allowing calculation of $D_{\text{m}}$ for the migration of the fluorescent probes within biofilms of various thicknesses. Effective diffusion coefficients for the size-fractionated FITC-conjugated dextrans were determined by using the one-dimensional form of the diffusion equation for homogeneous medium (30) as follows:

$$\frac{\partial C}{\partial t} = D_e \frac{\partial^2 C}{\partial z^2}$$

where $D_e$ is the diffusion coefficient (square centimeters per second), $z$ is the vertical distance ($z = 0$, h [any distance between 0 and h in centimeters]), $h$ is the thickness of the biofilm (centimeters), $t$ is time (seconds), and $C$ is the concentration (grey value). Initial conditions for the above differential equation were $C(z,0) = C_0$ 0 $< z < 2h$ and the boundary conditions were $C(0,t) = C_0 - \Delta C$ and $C(2h,t) = C_0 - \Delta C$. Applying the dimensionless time factor $T_p = T \cdot t = (4D_e \cdot t)/(2h)^2$, and substituting $F_{\text{p}}$ for $C_0$ and $\Delta C$ for $\Delta C$, then $D_e = [T_p \cdot (2h)^2]/4t$, where $T_p$ was estimated from a gray-level representation of Terzagli’s solution at time $t$ (13). Thus, using $T_p$, the depth of the biofilm, and the time required for $F/F_{\text{p}}$ to attain a specified value, the effective diffusion coefficient was calculated.

**Visualization.** After all diffusion studies were completed, bacteria and exopolymer in the biofilms were visualized by a negative staining technique involving the addition of 0.1% fluorescein to the irrigation solution (7). Biofilm depth was determined either by measuring distance above the attachment surface along transects in xz images with the Bio-Rad software on the Northgate 486 host computer or manually by using the computer-controlled focus motor while scanning in real time. All images of optical thin sections were printed with a Sony UP-5000W video printer.

**RESULTS AND DISCUSSION**

**Methodology.** Although incompletely characterized, the exopolysaccharide matrix of biofilms likely exists in a helical conformation, forming a three-dimensional network with pores and channels through which molecules can diffuse (12). This network may be viewed as a rigid gel in which the diffusion rate of the tracer molecule will decrease with its hydrodynamic radius, becoming zero at some cutoff which is related to the average mesh of the gel (23). The potential heterogeneity of the polymer matrix can complicate the estimation of diffusion rates through electrostatic and hydrophobic interactions with the probe molecules. However, if probes are selected to minimize these effects, it is possible to study diffusion in the biofilm by using a series of compounds of increasing molecular size. For our initial studies, we chose a series of hydrophilic, relatively inert, uncharged dextrans. The results of both FRAP and monitoring studies indicated that these probes did not interact extensively with the polymer matrix (i.e., recovery curves indicated nearly 100% recovery with the exception of the 2,000K dextran probe, which exhibited >80% recovery of fluorescence). In addition, the effective diffusion coefficient in the biofilm ($D_{\text{b}}$) and the relative diffusion coefficient ($D_{\text{aq}}/D_{\text{b}}$) of the dextrans were strongly size dependent, indicating that their diffusion was not complicated by transient binding or the effects of long-range forces in the polymer matrix (Table 1). Furthermore, these dextrans have been used previously in a wide range of diffusion studies in cell biology, mainly in conjunction with photobleaching techniques (16, 23, 28).

The established method for measuring diffusion coefficients in biological materials is FRAP (2, 16, 23, 28). The method involves the irreversible bleaching of fluorescently labeled molecules by brief exposure to intense light and monitoring the replacement of the bleached molecules with new fluorescent molecules. Bleaching occurs in the confocal plane and in a diabolo pattern above and below the focal plane, as described by Blonk et al. (3). The results obtained during a bleach and recovery experiment allow calculation of the rate of mobility (rate of fluorescence recovery) and the fraction of the molecular species that is mobile (degree of recovery). Although FRAP is commonly carried out with a laser attached to a standard optical microscope, the Bio-Rad MRC-600 can also be used (3). The theory of FRAP analysis has been described in detail before (2, 3, 16).

When FRAP was applied to biofilm matrices, we observed a satisfactory recovery of signal in photobleached regions, typically in the range of 90 to 100% (Fig. 2). We observed lower recovery of fluorescence with the 2,000K dextran, implying that cross-linkage of the matrix as a result of photodamage or interaction between the dextran and the matrix may have occurred, reducing mobility in the matrix. Concerns about photodamage artifacts resulting from the use of FRAP in cell biology have been raised previously (15). As generally used with light microscopy, FRAP also lacks the facility for producing high-quality images, which are important in biofilm studies because of the scale of the phenomena, the structure of the exopolymer matrix, and the nature and extent of the vertical and horizontal variability in biofilms (18). All of these factors indicated that a direct visualization technique would be the preferred means to study the mobilities of molecules within regions of microbial biofilms. Imaging methods combined with a monitoring beam should also yield detailed information on more complicated transport processes.

SCLM can obtain high-resolution images from a two- or three-dimensional area of known volume, at a known position, within biological materials (6, 7, 22, 29). It has also been shown

**TABLE 1. Molecular size parameters and diffusion characteristics of the fluorescent probes**

<table>
<thead>
<tr>
<th>Mol wt</th>
<th>$R_{\text{h}}$ (Å)</th>
<th>$R_{\text{c}}$ (Å)</th>
<th>$D_{\text{aq}}$ (cm$^2$s$^{-1}$)</th>
<th>$D_{\text{b}}$ (cm$^2$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>289</td>
<td>6.5</td>
<td>9.8</td>
<td>$3.3 \times 10^{-6}$</td>
<td>$7.7 \times 10^{-8}$</td>
</tr>
<tr>
<td>4,000</td>
<td>14.5</td>
<td>21.8</td>
<td>$1.48 \times 10^{-6}$</td>
<td>$3.1 \times 10^{-8}$</td>
</tr>
<tr>
<td>40,000</td>
<td>31</td>
<td>46.5</td>
<td>$0.70 \times 10^{-6}$</td>
<td>$1.7 \times 10^{-8}$</td>
</tr>
<tr>
<td>70,000</td>
<td>60</td>
<td>90.2</td>
<td>$0.36 \times 10^{-6}$</td>
<td>$1.1 \times 10^{-8}$</td>
</tr>
<tr>
<td>500,000</td>
<td>70</td>
<td>105.3</td>
<td>$0.31 \times 10^{-6}$</td>
<td>$1.1 \times 10^{-8}$</td>
</tr>
<tr>
<td>2,000,000</td>
<td>356</td>
<td>579.0</td>
<td>$0.056 \times 10^{-6}$</td>
<td>$0.7 \times 10^{-8}$</td>
</tr>
</tbody>
</table>

$R_{\text{h}}$, apparent hydrodynamic radius; $R_{\text{c}}$, radius of gyration; $D_{\text{aq}}$, diffusion coefficient in aqueous solution at 22°C; $D_{\text{b}}$, apparent diffusion coefficient in the biofilm at $23 \pm 2^\circ\text{C}$, 1 Å = 0.1 nm.
FIG. 3. Time series from 25 to 80 s, showing the variation in penetration of a 2,000K dextran within a mixed-species biofilm. Images are xy optical sections at a depth of 5 μm from the glass surface. Grey scale code: white, maximum intensity; black, undetectable.
that, when operated at minimum intensity, the laser beam has no apparent impact on growing bacterial cells and does not result in significant bleaching when used discontinuously at low intensity (7). Thus, SCLM has the requisite features for precise monitoring of events within the biofilm matrix.

The monitoring approach is simple in both concept and practice. A single pulse of medium containing fluorescent molecules is introduced into a flow cell, and a series of images are collected over time to record the migration of the fluorescent probe from the bulk phase through the hydrodynamic boundary layer and the intact biofilm (Fig. 3). The bulk phase may also be displaced with fluor-free medium, and the migration of the probe out of the biofilm can be monitored by the same method at the same position. The nature of image formation and collection in the SCLM created a series of images representing a well-defined volume element (optical cuvette) with known x, y, and z coordinates (6). One-dimensional diffusion coefficients were then calculated by measuring the rate of appearance or disappearance of the fluorescent molecules in the optical thin section being monitored. This procedure requires only that flow within the continuous-flow slide culture system be laminar and that the hydrodynamic boundary layer and biofilm remain undisturbed. It was also assumed that transport in the overlying medium was dominated by piston flow and thus that dispersion was negligible. This assumption was supported by the presence of laminar flow in the flow cell, as judged from the Peclet and Reynolds numbers calculated for the system. Previous studies have demonstrated that these requirements were met in the flow cell system used (20). In addition, the images presented in Fig. 4 and 5 indicate that the biofilm remained undisturbed by repeated monitoring experiments at the same location.

**Differential distribution of fluorescent probes in biofilms.**

The monitoring method provided a series of images showing the progressive appearance of the fluorescent probe within the monitored region. A typical time series for the appearance of a 2,000K dextran in a mixed-species biofilm at a location 5 μm from the glass (attachment) surface is shown in Fig. 3. After equilibrium was established, the differential penetration of biofilm regions by a specific probe could easily be defined by using grey-level variation within the image. A series of monitoring studies at the same location allowed evaluation of the differential penetration by FITC-conjugated dextrans of different sizes (Fig. 4). The percentage of the total area reaching the maximum grey level was a function of the molecular weight (i.e., the greatest penetration was achieved by the lowest-molecular-weight probes). The xz scanning function of the SCLM allowed the distribution of the probes throughout the entire depth of the biofilm to be assessed after equilibrium had been attained (Fig. 5). This view also provided considerable additional information about the relationship of the monitored locations to the overall biofilm architecture. For example, regions with high diffusion coefficients may be seen to be associated with pores or channels which are connected to the
FIG. 5. xz (sagittal) sections through a mixed-species biofilm showing penetration of the various dextrans into a 40-μm-thick biofilm. Note the extensive penetration into the vertical and horizontal channels within the biofilm.

FIG. 6. Curves showing the decrease in fluorescence after washout of the bulk phase. Note the effect of molecular weight on the profiles for the FITC-conjugated dextrans and fluorescein in a mixed microbial biofilm.

FIG. 7. Curves showing the effect of location on the rate of decrease in fluorescence at four different locations within a series of monitoring images with the 2,000K dextran.

bulk phase. Similarly, three-dimensional reconstructions of the biofilm location may be created by using a series of xy images through the biofilm, again providing an opportunity for three-dimensional assessment of the regions of restricted mobility for the probes. By imaging the distribution of probes of different molecular weights in the same biofilm location, we can map microzonal variation and the existence of microenvironments within biofilm communities. It may be necessary to examine a large number of microscope fields or a large area to obtain a representative value for any biofilm parameter. Korber et al. (18) indicated that areas as large as $6 \times 10^4 \mu m^2$ may be required to encompass all of the variability found in biofilms formed by *P. fluorescens*. Thus, in the application of...
this technique to any system, estimates of inherent variability and required sampling intensity may be necessary.

**Determination of effective diffusion coefficients.** The mobility of molecular species in biofilm systems is of great importance for understanding a number of dynamic processes, including transport of nutrients and wastes, effectiveness of antimicrobial agents, and the factors governing development of highly structured biofilm and bioaggregate communities (34). The series of grey-level images collected over a monitoring time course represent an extensive data set which defines changes in average image grey level with time as well as changes at any specific location within the image (Fig. 2 and 3). The shortest time interval that could be measured with the SCLM and our image acquisition program was 5 s; however, in the live-scan mode, acquisition time can be 5 ms. The change in average grey level between two time points reflects the action of diffusion of the fluorescent probe during the interim. Thus, plotting the change in grey level for diffusion into or out of the biofilm against time provides a graph of \( F/F_0 \) versus time. Our observations indicated that curves for diffusion into or out of the matrix gave the same results.

Typical results for fluorescence intensity curves are shown in Fig. 6. These results indicated that the method was sensitive to differences in the rates of diffusion for molecules of different molecular weights within the biofilm matrix, i.e., the biofilm mobilities of these probes scaled inversely with molecular size (Fig. 6). Diffusion coefficients varied with molecular weight (i.e., 4K dextran, \( D_w = 3.1 \times 10^{-8} \) cm² s⁻¹; 40K dextran, \( D_w = 1.7 \times 10^{-8} \) cm² s⁻¹; 70K dextran, \( D_w = 1.1 \times 10^{-8} \) cm² s⁻¹; and 2000K dextran, \( D_w = 0.7 \times 10^{-8} \) cm² s⁻¹). Table 1 shows a comparison of the \( D_c \) and \( D_m \) coefficients. The diffusion coefficient is also sensitive to the hydrated radius or the radius of gyration of the molecule. For example, the \( D_c \) values for the 70K and 500K dextran probes were virtually identical, a function of the similarity of their radii of hydration and gyration (Table 1). The FRAP technique gave similar results for \( D_c \) determinations. For example, we obtained a value of \( 0.51 \times 10^{-8} \) cm² s⁻¹ for diffusion of the 2000K dextran in the mixed-species biofilm. A comparison of the results obtained for the pure culture of *P. fluorescens* shows that the \( D_m \) values for the 2000K dextran and fluorescein were \( 0.26 \times 10^{-8} \) and \( 0.96 \times 10^{-8} \) cm² s⁻¹, respectively, showing a trend based on molecular weight similar to those in the mixed-species biofilm although quantitatively different. These results implied that the technique was also sensitive to differences in biofilm composition.

As shown in Fig. 3, 4, and 5, the mixed-species biofilm exhibited spatial heterogeneity with respect to penetration by the various probes. Figure 7 shows the range of fluorescence intensity curves generated for the 2000K dextran probe when subsets of an image were selected for analysis. It is apparent that \( D_c \) values for a specific probe may vary by a large degree in a mixed-species biofilm, depending on the location selected. Thus, depending on the degree of apparent heterogeneity shown within the biofilm, the selection of a location(s) or the analysis of subsections of the image may be required to allow estimation of the variability in \( D_c \) values for a specific probe. Techniques for assessing sampling requirements in biofilms have been discussed previously (18).

In all cases, the biofilm diffusion coefficients were less than those for the molecules in bulk solution, indicating a degree of hindered diffusion through the biofilm matrix. Measurements of diffusion coefficients indicated that a 70K dextran had a \( D_w \) of \( 4.0 \times 10^{-7} \) cm² s⁻¹ in water (\( D_w \)), whereas in the biofilm matrix, \( D_w (D_m) \) was estimated to be \( 1.1 \times 10^{-8} \) cm² s⁻¹. In general, measurements of diffusion coefficients, determined through indirect methods, for various molecules (e.g., oxygen, glucose, ammonia, nitrate, sodium, and bromide) through biofilms and bioflocs are 50 to 80% of their respective values for water (9). However, some authors (5) reported a large decrease in the diffusion coefficient of oxygen from \( 8.3 \times 10^{-6} \) cm² s⁻¹ in the medium to \( 4.0 \times 10^{-7} \) cm² s⁻¹ in the biofilm matrix. The diffusion rates of the dextrans in the biofilm measured in the present study were 2.4 to 12.5% of the respective diffusion rates in water. A comparison of the mobility of fluorescein in 1.5% agar and biofilm material shows that the \( D_m \) was \( 7.7 \times 10^{-8} \) cm² s⁻¹ whereas in the gel the \( D_w \) was \( 4.7 \times 10^{-9} \) cm² s⁻¹ (33), indicating that diffusion in biofilm materials was slower than that in agar.

The effective diffusion rate of a solute in a pore space or gel network is frequently less than its value in the bulk solution (4, 23). This phenomenon is characterized as hindered or restricted diffusion and reflects the degree of interaction between the diffusing molecule and the matrix. Luby-Phelps et al. (24) demonstrated hindered size-dependent diffusion of a variety of fluor-conjugated dextrans in cytoplasm. These results showed trends similar to those obtained in the present study. Studies of mobility in cytoplasm have shown that macromolecules such as bovine serum albumin, insulin, and apoferritin exhibit diffusion coefficients in the range from \( 1 \times 10^{-8} \) to \( 2 \times 10^{-9} \) cm² s⁻¹, indicating hindered diffusion in this biological matrix (15). Thus, the results obtained with the method described in this article for determining diffusion coefficients of biofilm matrices are in general agreement with the results obtained by other methods. Furthermore, the behavior of the dextrans followed the molecular weight- or radius of gyration-dependent trends shown for these molecules in previous studies of diffusion in cell cytoplasm.

Monitoring with an SCLM can be used to assess the fate of a wide range of fluorescent probes in the biofilm matrix, including proteins, dextrans, Ficolls, latex spheres, and others. The method allowed demonstration of size-dependent diffusion, hindered diffusion, and spatial variability in effective diffusion coefficients within biofilms. Application of FRAP allowed estimation of the degree of interaction between probe and matrix. Analysis of the mobility of these analogs within the biofilm can serve as an indicator of diffusion and flow, as well as the polymerization state, apparent viscosity, and limiting equivalent porosity of the exopolymer. In situ monitoring also allowed image acquisition, appropriate scale of study, and both \( x \) and \( z \) monitoring. Three-dimensional reconstructions of probe penetration studies may also be made. Finally, this approach provides a method for understanding the structure and function of the exopolysaccharide matrices of intact, fully hydrated biofilms.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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**Table 1**

<table>
<thead>
<tr>
<th>Molecule</th>
<th>( D_m ) (cm² s⁻¹)</th>
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<tr>
<td>4K dextran</td>
<td>3.1 \times 10^{-8}</td>
</tr>
<tr>
<td>40K dextran</td>
<td>1.7 \times 10^{-8}</td>
</tr>
<tr>
<td>70K dextran</td>
<td>1.1 \times 10^{-8}</td>
</tr>
<tr>
<td>2000K dextran</td>
<td>0.7 \times 10^{-8}</td>
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</table>

**Figures**

1. **Fig. 1.** Schematic of the experimental setup.
2. **Fig. 2.** Examples of grey-level images from the biofilm at different times.
3. **Fig. 3.** Fluorescence intensity curves for 4K dextran in water.
4. **Fig. 4.** Fluorescence intensity curves for 4K dextran in the biofilm.
5. **Fig. 5.** Fluorescence intensity curves for 4K dextran in agar.
6. **Fig. 6.** Fluorescence intensity curves for 4K dextran in the mixed-species biofilm.
7. **Fig. 7.** Range of fluorescence intensity curves generated for the 2000K dextran probe when subsets of an image were selected for analysis.

**Additional Figures**

- Three-dimensional reconstructions of probe penetration studies.
- In situ monitoring of diffusion in cell cytoplasm.
probe techniques for determining diffusivities and respiration rates in 