

## Diffusion of Macromolecules in Model Oral Biofilms<sup>∇</sup>

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**The diffusive penetration of fluorescently tagged macromolecular solutes into model oral biofilms was visualized by time-lapse microscopy. All of the solutes tested, including dextrans, proteases, green fluorescent protein, and immunoglobulin G, accessed the interior of cell clusters 100 to 200  $\mu\text{m}$  in diameter within 3 min or less.**

The diffusive properties of macromolecular solutes in biofilms remain poorly understood even though the diffusion of these molecules is a critical aspect of the growth, pathogenicity, and control of dental plaque and other biofilms. Examples of phenomena that are likely directly impacted by macromolecule diffusion include the following: (i) antibody access to biofilm and the ensuing immune response, (ii) penetration of antimicrobial peptides into biofilms, (iii) gene transfer via extracellular DNA, (iv) access of antimicrobial enzymes and enzymes that degrade extracellular polymeric substances, and (v) nutrient delivery into biofilms in the form of polysaccharides and proteins.

There is little consensus on the values of effective diffusion coefficients ( $D_e$ s) of macromolecules in biofilms (see Fig. 5 of reference 8). Consider, for example, the transport of dextrans, whose diffusion in biofilms has been the subject of multiple studies, probably because fluorescently tagged dextrans are readily commercially available. Reported values of diffusion coefficients for dextrans with molecular weights (MW) of 4,000 to 10,000 in bacterial biofilms, from four independent studies, are (in units of  $\mu\text{m}^2 \text{s}^{-1}$ ) 0.69, 3.1, 43.8, and 196 (12). This range spans a 280-fold difference in the estimated diffusion coefficient. The protein phycoerythrin (MW, 240,000) has been reported to have a relative effective diffusivity of 0.59 in a *Pseudomonas/Klebsiella* biofilm (3), whereas Thurnheer et al. (12) reported a value of 0.002 in a six-species oral biofilm.

The purpose of this study was to directly visualize the penetration of a variety of macromolecules into model oral biofilms and to estimate diffusion coefficients. This was done using time-lapse confocal scanning laser microscopy followed by image analysis.

Using time-lapse confocal scanning laser microscopy, we visualized the diffusive penetration of fluorescently tagged macromolecules into three-species biofilms formed by oral microorganisms grown in a flow cell. The technique was essentially that described by Rani et al. (6). Three-species biofilms of *Streptococcus oralis* ATCC 10557, *Streptococcus gordonii* ATCC 10558, and *Actinomyces naeslundii* ATCC 19039 were

developed as detailed by Takenaka et al. (10). When the fluorescent probe solution was introduced into the capillary, it immediately surrounded cell clusters and then progressively migrated further into the cell cluster in a radially symmetric fashion. Fluorescence in the interior of the biofilm plateaued and remained steady. The mean radial dimension of cell clusters was  $82 \pm 34 \mu\text{m}$ .

Image analysis was applied to quantify the fluorescence intensity at the center of a biofilm cell cluster and the fluorescence intensity in the bulk fluid outside the cluster. The fluorescence intensity at the cluster center was divided by the intensity in the fluid outside the cluster, and this ratio was plotted in time; examples of the resulting data sets are given in Fig. 1. These results are from sequential experiments performed on the same biofilm cell cluster. This comparison immediately makes it clear that a small solute, such as fluorescein (Fig. 1, top panel), diffuses into the center of the cluster faster than does a larger solute, such as dextran (Fig. 1, third panel).

The diffusive penetration time was quantified as the time required to attain, at the center of a biofilm cell cluster, 90% of the equilibrium fluorescence intensity. This parameter is denoted by  $t_{90}$ , and all of the experimentally measured values are reported in Table 1. The mean value (and standard deviation) of  $t_{90}$  was as short as 13 s for 3,000-MW dextran and as long as 190 s for immunoglobulin G (IgG). The penetration time depended on the size of the cell cluster. Penetration times were longer in larger cell clusters.

Because penetration time is an extrinsic parameter that depends on the shape and size of a particular cell cluster, we sought to estimate the value of the  $D_e$  in the biofilm, an intrinsic parameter. These estimates were calculated as  $D_e = 0.31R^2/t_{90}$  (9), where  $R$  is the cluster radius, and are summarized in Table 2. The  $D_e$  in biofilm ranged from 22% of its value in pure water for IgG to 90% of the aqueous diffusion coefficient ( $D_{\text{aq}}$ ) for the 3,000-MW dextran.

Fluorescently tagged macromolecular solutes readily accessed the interior of biofilm cell clusters developed in an in vitro flow cell system. None of the macromolecules investigated, including the largest, IgG, failed to reach the center of microbial cell clusters that were approximately a few hundred micrometers in diameter. The time for solutes of sizes from a few thousand Daltons to 100,000 Da to reach the center of these cell clusters was on the order of tens of seconds to 3 min. These penetration times corresponded to  $D_e$ s in the biofilm

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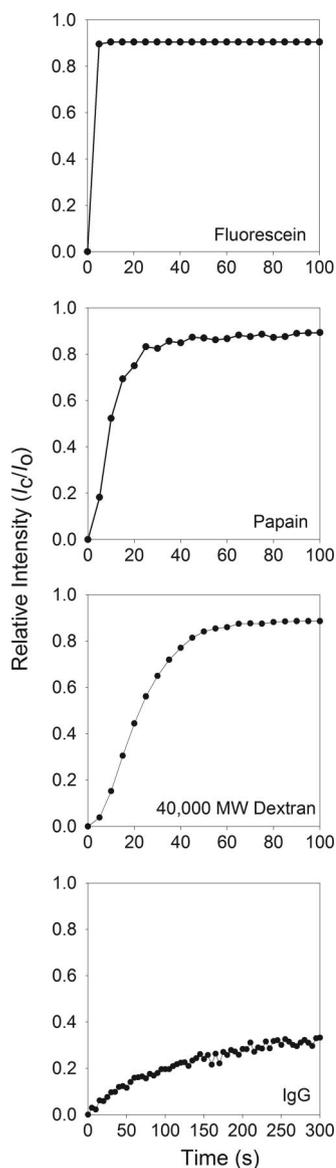


FIG. 1. Typical time course of fluorescent probe penetration into a biofilm cell cluster. The relative intensity is the fluorescence intensity at the center of the cluster divided by the intensity in the fluid outside the cluster. These data are from sequential experiments with the same cell cluster.

that were between 22% and 90% of the respective diffusion coefficient in pure water. The relative effective diffusivity decreased with increasing molecular weight (Fig. 2).

The  $D_e$  of fluorescein has been measured in a few other in vitro biofilm studies. The relative  $D_e$  for fluorescein ranges widely, with reported values of 0.023 (4), 0.97 (3), 0.91 (2), and 0.32 (6). Our measurement in the present study of a relative  $D_e$  of 0.40 is close to the value of 0.32 that was found in an earlier study done by our group using *Staphylococcus epidermidis* biofilms (6). Some of the variation from one study to the next may be explained by different degrees of sorption of the dye to components of the biofilms.

One puzzle posed by these results is why the relative  $D_e$  for fluorescein (0.40) is smaller than the relative  $D_e$ s for the much

TABLE 1. Diffusive penetration times of various macromolecular solutes in model oral biofilms<sup>a</sup>

Cluster	R (μm)	$t_{90}$ (s)										
		F	D, 3K	D, 10K	D, 40K	D, 70K	Papain	Ficin	GFP	ConA	IgG	
1	147	20	25	55	90	105						195
2	143	20	20	50	105	145						150
3	51	5	10	30	45	45	25					
4	44	5	5	35	70	70	45					
5	120	10	15	40	85	85	50					
6	80	15	15	45	95	125						
7	66	10	15	25	80	155	35					
8	88	10	15	15	60	145						
9	80	15	15	15	55	155						
10	65	5	15	25	50	155						
11	67	5	15	25	30	50						
12	60	5	5	25	30	30						
13	52	5	5	25	30	30						
mean		10	13	31	66	99	26					

<sup>a</sup> Abbreviations: F, fluorescein; D, dextran; GFP, green fluorescent protein; ConA, concanavalin A. Molecular weights in thousands (K) are given for dextran.

TABLE 2. Estimated  $D_e$  values of various macromolecular solutes in model oral biofilms<sup>a</sup>

Solute	MW (g/mole)	$D_e$ ( $\mu\text{m}^2 \text{s}^{-1}$ )	$D_{\text{aq}}$ ( $\mu\text{m}^2 \text{s}^{-1}$ )	$D_e/D_{\text{aq}}$	No. of measurements
Fluorescein	376	219 $\pm$ 103	540	0.40	13
Dextran	3,000	167 $\pm$ 83	186	0.90	13
	10,000	78 $\pm$ 47	108	0.72	13
	40,000	34 $\pm$ 19	55	0.62	13
	70,000	26 $\pm$ 18	47	0.56	13
Papain	23,000	70 $\pm$ 39	96	0.73	7
Ficin	25,000	64 $\pm$ 26	94	0.68	7
GFP	27,000	66 $\pm$ 65	87	0.76	5
ConA	104,000	26 $\pm$ 11	58	0.57	6
IgG	150,000	10 $\pm$ 7	44.5	0.22	6

<sup>a</sup> GFP, green fluorescent protein; ConA, concanavalin A.

larger fluorescein-tagged dextrans (0.56 to 0.90). Intuition suggests that the relative  $D_e$  should decrease with increasing molecular weight. This difference may be due to greater sorption of fluorescein, perhaps through electrostatic interactions, to components of the biofilm. Fluorescein was the first solute tested on each biofilm, so it may have occupied some binding sites and thus slightly reduced sorption of subsequently tested solutes. Sorption retards diffusive penetration and reduces the relative  $D_e$  (7). We note that the value of the fluorescein relative  $D_e$  determined in this study is close to the mean value for solutes with MW greater than 45, 0.39, reported in a review (8).

Published measurements of the relative  $D_e$ s in model oral biofilms or harvested dental plaque are compared in Fig. 2. The strongest impression made by this comparison is a lack of consensus, the situation noted in the introduction. For example, our measurement of the relative effective diffusivity of 40,000-MW dextran, 0.62, is in good agreement with the value reported by Birmingham et al. (1), 0.61, but is more than an order of magnitude larger than the value 0.017 reported by Thurnheer et al. (12). Here we consider three possible pitfalls of experimental measurements of diffusion in biofilms.

The first pitfall is inherent in techniques that average over a large (compared to the dimension of biofilm structural features, such as clusters and channels) area. Such would be the case with a diffusion half-cell or with attenuated total reflection

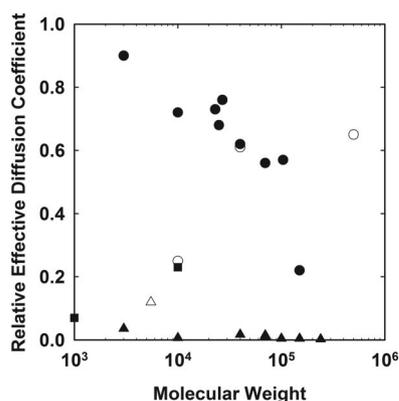


FIG. 2. Comparison of relative  $D_e$ s of macromolecules measured in dental plaque or in in vitro oral biofilms. Sources: ●, this study; ○, reference 1; ▲, reference 12; ■, reference 5.

spectroscopy. These techniques effectively average the diffusive properties within cell clusters and water-filled pores. As Marcotte and coworkers (5) point out, if a solute is partially excluded from the biofilm, these techniques will weight the average diffusion coefficient even further toward its value in the pores or channels of the biofilm. This pitfall therefore results in overestimation of the diffusion coefficient within a biofilm cell cluster itself. The technique employed in the present investigation avoids this pitfall by focusing the transport analysis exclusively within independently identified cell clusters.

The second pitfall is the possibility that convective transport, or bulk fluid flow, enhances movement of the solute. This artifact will result in an overestimate of the diffusion coefficient. Three observations argue against any interference of convection with measurements in our investigation. First, the ingress of fluorescent probes was the same on upstream and downstream edges of clusters. Second, if convection were the dominant transport process, the penetration time would be the same for every solute, since it would be determined by the fluid velocity rather than the molecular diffusion coefficient. This was not the case with our data, which clearly do show a dependence on the size of the probe. Third, we did some experiments with fluorescein in which the flow of the dye solution was maintained throughout the observation period instead of the standard procedure in which the flow is stopped once the dye solution has completely filled the capillary tube. No difference in the measured diffusion times was observed using these two protocols. We did occasionally identify cell clusters, usually very large ones, that appeared to contain channels allowing fluid flow. Such clusters were not used for diffusion measurements.

The third and probably most serious pitfall is the inadequate treatment of external mass transfer resistance. This refers to the possibility that diffusive penetration is slowed by the requirement for the solute to pass through a layer of stagnant or slow-moving fluid outside of the biofilm cell cluster. All of the studies whose data are reported in Fig. 2, including ours, have neglected external mass transfer in the analysis of results. Neglecting external mass transfer can result in underestimation of the actual diffusion coefficient in the biofilm. Because the degree to which external mass transfer impinges on the validity of the measurement is a complex integration of system geometry and hydrodynamics, it is usually difficult to evaluate whether an artifact is present from the information provided in a journal article. In our own study, we have direct visual evidence that external mass transfer does not significantly influence our results. If external mass transfer resistance is significant, it will give rise to a concentration gradient in the fluid surrounding the cell cluster. Our technique allows this concentration to be determined via the fluorescence intensity in the fluid near the cluster. No gradient in the fluorescence signal or decay of fluorescence was observed in the fluid around biofilm clusters. Also, we note that our estimates of the relative diffusion coefficient are generally larger than those of other groups (Fig. 2). We conclude that it is unlikely that we have underestimated the actual diffusion coefficient.

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