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Daptomycin Rapidly Penetrates a *Staphylococcus epidermidis* Biofilm▽†

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Fluorescently tagged daptomycin accessed the interior of *Staphylococcus epidermidis* biofilm cell clusters within minutes. The diffusion coefficient of daptomycin in the biofilm was 28% of its value in pure water. Daptomycin activity against staphylococci embedded in biofilms is unlikely to be limited by penetration of the antibiotic into the biofilm.

Infections associated with microbial biofilms that form on implanted medical devices are notoriously difficult to resolve with antimicrobial chemotherapy (9). One of the obvious and oft-mentioned explanations for the failure of antibiotic treatment is that the antibiotic may not penetrate the biofilm. If the time required for an antibiotic to penetrate the biofilm is long compared to the treatment duration, then slow penetration is a plausible explanation for the observed antibiotic tolerance of the biofilm. On the other hand, if the time required for an antibiotic to penetrate the biofilm is short compared to the treatment time, then biofilm penetration is probably not the limiting step. In this latter case, biological explanations for biofilm tolerance should be sought.

There are few direct experimental visualizations of antibiotic penetration into bacterial biofilms. Stone et al. imaged the rapid delivery of tetracycline into thin *Escherichia coli* biofilms (10). This drug penetrated biofilms that were approximately 15-μm thick within 10 min. Jefferson et al. demonstrated that vancomycin partially permeated a *Staphylococcus aureus* biofilm during 1 h of exposure to the drug under static conditions (4).

The purpose of the work reported in this article was to determine the time course of diffusive penetration of daptomycin into large, dense clusters of staphylococcal biofilm.

Biofilms of *S. epidermidis* strain RP62A (ATCC 35984) were grown in a flow cell at 37°C with continuous flow of 1/10 strength tryptic soy broth (7). Time-lapse confocal scanning laser microscopy was then used to analyze diffusive penetration of a fluorescent solute as described previously (7). Daptomycin was fluorescently tagged by adding aqueous $NaHCO_3$ and either Bodipy or rhodamine to daptomycin dissolved in dimethylformamide. Fluorescently labeled daptomycin was dissolved in a phosphate buffer containing 2 mM Mg^{2+} to a final concentration of 40 μg ml^{-1} and pumped through the capillary biofilm reactor at a flow rate of 1 ml min^{-1}. Diffusion measurements were obtained inside isolated biofilm clusters attached to the ceiling of the glass capillary, at a focal plane approximately 10 μm from the glass surface. Diffusion experiments were conducted at an ambient temperature of approximately 23°C.

S. epidermidis formed dense, heterogeneous biofilms in glass capillary tube reactors during the 20-h growth period. Thick biofilm formed in the corners of the flow cell and also occasionally as clusters in the middle of the tube walls. Diffusion experiments were conducted on the relatively isolated clusters. The radial dimension of cell clusters (R) ranged from approximately 100 to 200 μm. The overall mean radius and standard deviation was 158 ± 48 μm.

When fluorescent-tagged daptomycin was introduced into the flow cell, fluorescence first developed in the bulk fluid. The drug stained the periphery of cell clusters and then progressively moved inward toward the center of the cluster. Similar behavior was observed for the Bodipy-FL- and rhodamine-tagged versions of the antibiotic. An example of one of these experiments is presented in Fig. 1, and videos of two of the experiments, both 6 minutes in real time, are posted as supplemental material.

No alteration of biofilm structure occurred during exposure to the antibiotic in any of the experiments. This was evident from the stability of the structures as observed in transmission mode imaging.

Image analysis comparing the fluorescence intensity at the center of a cell cluster with the intensity in the surrounding bulk fluid (7) resulted in a characteristic S-shaped curve. The time required for the fluorescence intensity in the center of the biofilm cluster to reach 50% of the steady-state maximum bulk fluid fluorescence intensity was determined and designated t_{50}. These penetration times are summarized in Table 1. The t_{50} penetration times ranged from a fraction of a minute to a few minutes, with an overall mean and standard deviation of 91 ± 71 s. Penetration time increased with increasing cluster radii. The experiments demonstrated that the antibiotic penetrated freely into the biofilm clusters.

The estimated aqueous diffusion coefficients of the Bodipy- and rhodamine-labeled daptomycins at 23°C are 1.73×10^{-6} cm^2 s^{-1} and 1.78×10^{-6} cm^2 s^{-1}, respectively (5, 6). The estimated aqueous diffusion coefficient of unlabeled daptomycin at the same temperature is 1.96×10^{-6} cm^2 s^{-1}. The effective diffusion coefficient (D_e) of labeled daptomycin in the biofilm was estimated from the t_{50} values by using the relationship $D_e = 0.139R^2/t_{50}$. The effective diffusion coefficients (and standard deviations) of the Bodipy- and rhodamine-tagged an-

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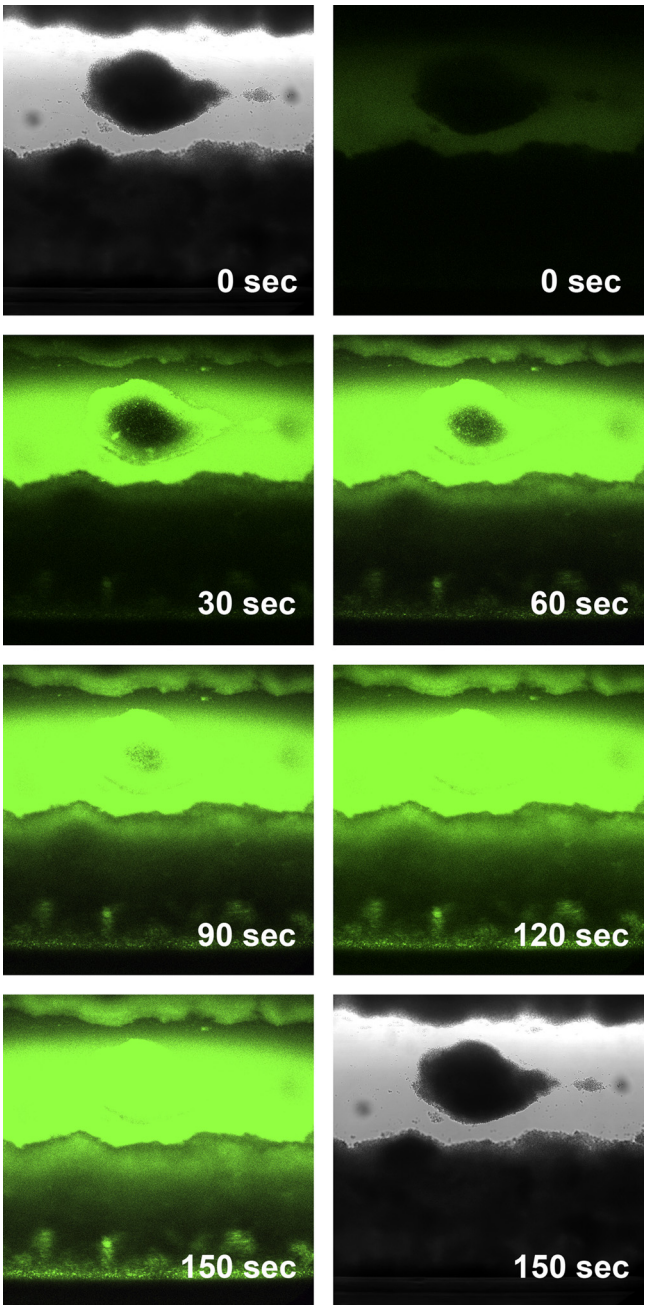

FIG. 1. Transient diffusion of Bodipy-tagged daptomycin (green) into an *S. epidermidis* biofilm cell cluster (dark in grayscale) growing in a flow cell. The time indicated is the elapsed time after introduction of the antibiotic into the system. The upper-left and lower-right panels are transmission images; these reveal the distribution of biomass (dark) and show that the biofilm structure was unchanged during the course of the diffusion experiment. All other panels represent fluorescence in the green channel at the same location; these reveal the progressive inward diffusion of daptomycin. Flow is from left to right. Each panel is 750 μm on each side.

tibiotics in biofilm were 5.2 ± 2.6 × 10^{-7} cm^2 s^{-1} and 4.7 ± 2.9 × 10^{-7} cm^2 s^{-1}, respectively. These values correspond to 30% and 26% of the estimated values in pure water, respectively.

Taking the mean relative effective diffusion coefficient of daptomycin in bacterial biofilm to be 28% of its value in pure water, the estimated effective diffusion coefficient of unlabeled daptomycin in staphylococcal biofilm at 37°C is 0.78 × 10^{-6} cm^2 s^{-1}.

The equilibrium value of the fluorescence intensity ratio of the biofilm to bulk fluid signal was consistently close to one (Table 1), suggesting that there was not much sorption of the antibiotic to the biofilm.

Daptomycin can bind to serum proteins and can form micellar structures in the presence of Ca^{2+} or Mg^{2+} ions (3). Either of these interactions would be expected to retard the diffusion of the drug. There was no protein in our in vitro system, but there was excess magnesium.

In this study, we have demonstrated that fluorescent-tagged daptomycin can readily penetrate thick *S. epidermidis* biofilms. The time scale for biofilm penetration is on the order of a minute or two. This is much shorter than the duration of antibiotic exposure, which is typically tens of hours. Slow penetration is therefore not likely to be an issue in the activity of daptomycin in staphylococcal biofilms. Of course, this conclusion is tempered by the fact that this result is from an in vitro model rather than an in vivo model and that the work was performed with a single bacterial strain. Viability of the clusters was not measured, because the capillary flow cell reactor used in this study is designed for optimal microscope imaging but is poorly suited to viability measurements. We suggest that daptomycin should be evaluated for antibacterial activity in biofilms.

The overall outcome demonstrated in this project of adequate antibiotic penetration concurs with several published studies of antibiotic or tracer penetration into staphylococcal biofilms (1, 2, 7, 11, 12). These prior studies were performed with rifampin (rifampicin), vancomycin, ofloxacin, and cefotiam.

The relative effective diffusivity estimated in this work for daptomycin in biofilm of 28% of the diffusion coefficient in pure water is similar to published values. Stewart has suggested a consensus value of 25% for the relative effective diffusivity for most organic solutes (8).

TABLE 1. Summary of biofilm dimensions and observed t_{50} for fluorescently tagged daptomycin[a]

Date in 2007	Tag	R_1 (μm)	R_2 (μm)	R_{avg} (μm)[b]	t_{50} (s)[c]	I/I_o
17 May	Bodipy	181	103	142	67	1.11
23 May	Bodipy	278	168	223	210	1.44
13 June	Bodipy	223	153	188	60	1.39
10 May	Rhodamine	217	115	166	140	1.00
17 May	Rhodamine	207	97	152	40	1.00
17 May	Rhodamine	94	63	79	26	1.00

[a] R_1 is the radial dimension of a cell cluster measured on its long access; R_2 is the dimension of the cluster measured in the orthogonal direction; R_{avg} is the average radial dimension; t_{50} is the penetration time; and I/I_o is the relative intensity of the equilibrium staining in the biofilm compared to that in the bulk fluid. The uncertainties are standard deviations.

[b] The mean of the values in the top three rows is 184 ± 41 μm, and that of those in the bottom three rows is 132 ± 47 μm.

[c] The mean of the values in the top three rows is 112 ± 85 s, and that of those in the bottom three rows is 69 ± 62 s.

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