Optical reflectance assay for the detection of biofilm formation

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Abstract. We describe the protocol for an inexpensive and nondestructive optical reflectance assay for the measurement of biofilm formation. Reflectance data are obtained using an Ocean Optics (Dunedin, Florida) USB 2000 spectrometer with a polychromatic light source. A fiber optic cable is used both for illumination and collection, and Ocean Optics OOIBase32 Platinum software is used for preliminary processing of the data. Differences in reflectance data collected at times ranging from 2 to 24 h distinguish between cell attachment and volume growth for two strains of *Enterococci*. Confocal scanning laser microscopy imaging is used to confirm these results. Phase contrast microscopy images are also obtained in conjunction with reflectance measurements for several different biofilm specimens. The experiments consider biofilm formation on glass and polystyrene substrata, but the method can be used for many other abiotic substrata of interest, both opaque and nonopaque. [DOI: 10.1117/1.1953347]

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1 Introduction

A biofilm consists of a population of microbial organisms that are encased within a matrix of organic materials generated by the microbes themselves.[1,2] Biofilms appear to be widespread in the environment[3] and, as a consequence, play a significant role in the persistence of microbes where otherwise harsh conditions would prevent long-term survival.[2] Potential advantages conferred by this mode of existence include an improved ability to survive dessication, ultraviolet light, extreme variations in pH, chemical oxidation, and offensive agents such as antibiotics.[4]

Biofilms have an enormous impact on human health. According to the National Institutes of Health, more than 80% of infections involve biofilms, many of which are acquired in hospitals. These infections take their toll in both loss of life and financial cost.[5] Biofilms are integral to both water-borne and food-borne diseases. It has long been known that biofilms exist in drinking water distribution systems and they continue to exist despite efforts to eliminate them. While most bacterial biofilms in drinking water distribution systems are nonpathogenic, pathogens can invade these structures and take advantage of the protection afforded by them. In addition, long-term treatment of biofilms with chlorine can lead to chlorine-resistant strains.[6] The Centers for Disease Control estimate that 250 different diseases are transmitted by food consumption. Food contamination can occur in food-processing environments where bacteria are associated with biofilms. For example, the Gram-positive bacterium *Listeria monocytogenes*, which forms biofilm on stainless steel and other surfaces, is able to replicate in refrigerated, ready-to-eat foods and is associated with food-borne diseases with relatively high mortality rates.[7] As with other bacteria, there is a large variance in the ability of any single *L. monocytogenes* strain to form a biofilm.[8]

The standard assay for measuring biofilm formation is the crystal violet (CV) assay, which involves quantification of dye bound to cells within a biofilm on a polystyrene substratum.[9] The CV assay can be used to detect biofilm formation on other types of substrata, but the method for doing so is indirect. In addition, the CV assay requires multiple washings, possibly resulting in loss of cells, and it requires destruction of the biofilm. Scanning electron microscopy (SEM) has also been used to examine biofilm formation. SEM, however, is expensive, and data can be difficult to quantitate.

A number of optical techniques have been developed previously for use with biofilms. Among these are light microscopy,[10,11] infrared spectroscopy,[12] reflectance spectroscopy,[13] confocal scanning laser microscopy,[13] and optical fluorometry.[14,15] We present the protocol for a new, inexpensive, nondestructive optical reflectance assay for measuring biofilm formation. The reflectance assay is a semiquantitative method that can be used with numerous abiotic surfaces, both opaque and nonopaque; it has the potential for use on certain types of biotic surfaces as well. Time series reflectance measurements can be made of growing biofilms as well as of biofilms that have been fixed. Graphs of reflectance as a function of wavelength provide information on the state of biofilm formation and indicate whether or not a bacterium is capable of forming a biofilm. Moreover, the reflectance data

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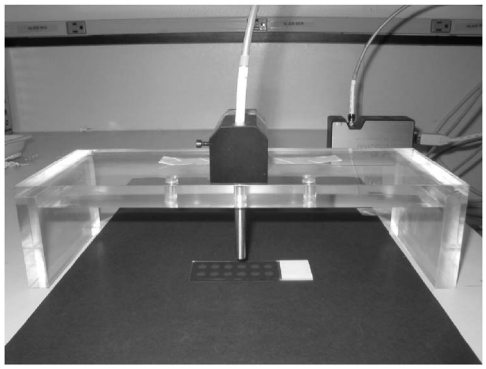


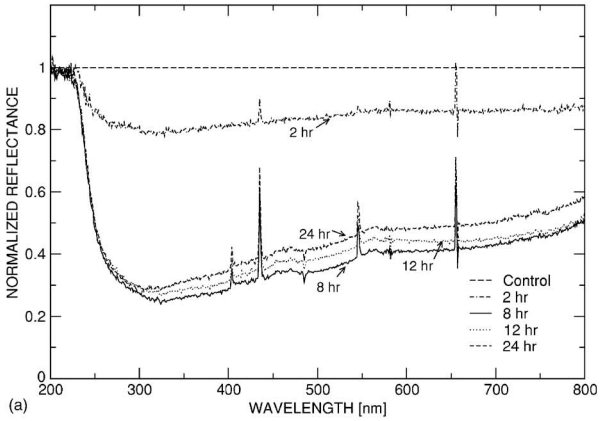
Fig. 1 Optical fiber probe set-up. The spectrometer is the box partially hidden at the back. The light source is not shown.

can be averaged to provide a single number that permits a yes-or-no answer to the question of whether a biofilm has formed. Thus, the reflectance assay can be used for rapid determination of biofilm formation, which is useful for screening mutant libraries and assessing phenotypic variation among isolates. Optical reflectance measurements include both cell and exopolysaccharide (EPS) volume. It is possible that the data can be analyzed to determine the percentage of each, but this remains the subject of further research. Finally, biofilm specimens used with the reflectance assay can be fixed for long-term archiving. Images have been obtained of specimens stored for six months with no apparent degradation.

2 Optical Reflectance Measurements

The measurement system consists of an Ocean Optics USB 2000 spectrometer (Dunedin, Florida) and a UV2/OFLV-4 fiber optic cable attached to a DT 1000CE deuterium tungsten halogen light source. The light source provides the continuous spectrum of a deuterium ultraviolet light source and a tungsten-halogen visible/shortwave near-infrared light source in one optical path. The fiber optic cable is used both for illumination and collection. Data are collected on a Windows-based PC running the Ocean Optics OOIBase32 Platinum software package. The user's guide provided with the hardware and software explains their basic usage, but to obtain accurate biofilm measurements it is necessary to follow the steps decribed next.

The power supply must be turned on at least 30 min prior to making reflectance measurements to prevent spurious spikes in the reflectance profiles. As shown in Fig. 1, the optical fiber probe should be placed perpendicular to the substratum to give normally incident light. In this figure, the substratum is a standard $25 \times 75 \times 1$-mm glass slide with Teflon masking, which delineates 12 independent wells on the slide. The probe distance, the distance from the tip of the probe to the scanning surface, must be adjusted so the incident light illuminates approximately 80% of the well. For a well 6 mm in diameter, this is between 5 and 5.4 mm. The probe distance will depend on both the thickness of the substratum and the diameter of the well, but with shorter distances there will be less beam spreading and more uniform light coverage. A thick sheet of black matte paper should be placed under the substratum to absorb both transmitted and extraneous scattered light.



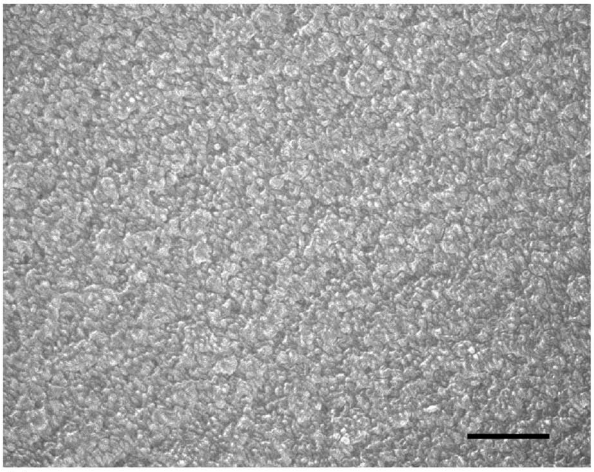


Fig. 2 (a) Normalized reflectance measurements as a function of wavelength for Bfm+ *E. faecalis* isolate on glass at times $t=0$ (control), 2, 8, 12, and 24 h. (b) Phase contrast microscope image of this isolate on glass at 24 h. The scale bar is 50 μm.

Reflectance measurements are collected following the OOIBase32 spectrometer software instructions provided in the Reflection Experiments section. *Store Reference* and *Store Dark* are used to standardize the scanning procedure. *Store Reference* is used to set the maximum reflectance value and *Store Dark* to establish a minimum baseline. The maximum reflectance value is obtained by scanning a control well (explained later) and adjusting the integration time until the maximum signal is approximately 3500 counts (this number will fluctuate) while in scope mode. Reflection intensity differs appreciably for different substrata, and the greater the reflectance, the shorter the integration time. After the maximum reflectance value has been obtained and stored, the light path should be blocked (by covering the end of the optical fiber probe) and the resulting (flat) dark spectrum stored. If noisy spikes occur in the reflectance signal, the room lights may have to be dimmed.

Before a particular type of substratum is used in a biofilm experiment, it must be examined for possible anomalous reflectance behavior. For example, reflectance might depend on the orientation of the surface relative to the light source. If this is the case, the surface must be oriented in the same direction for all biofilm measurements. To characterize a sub-

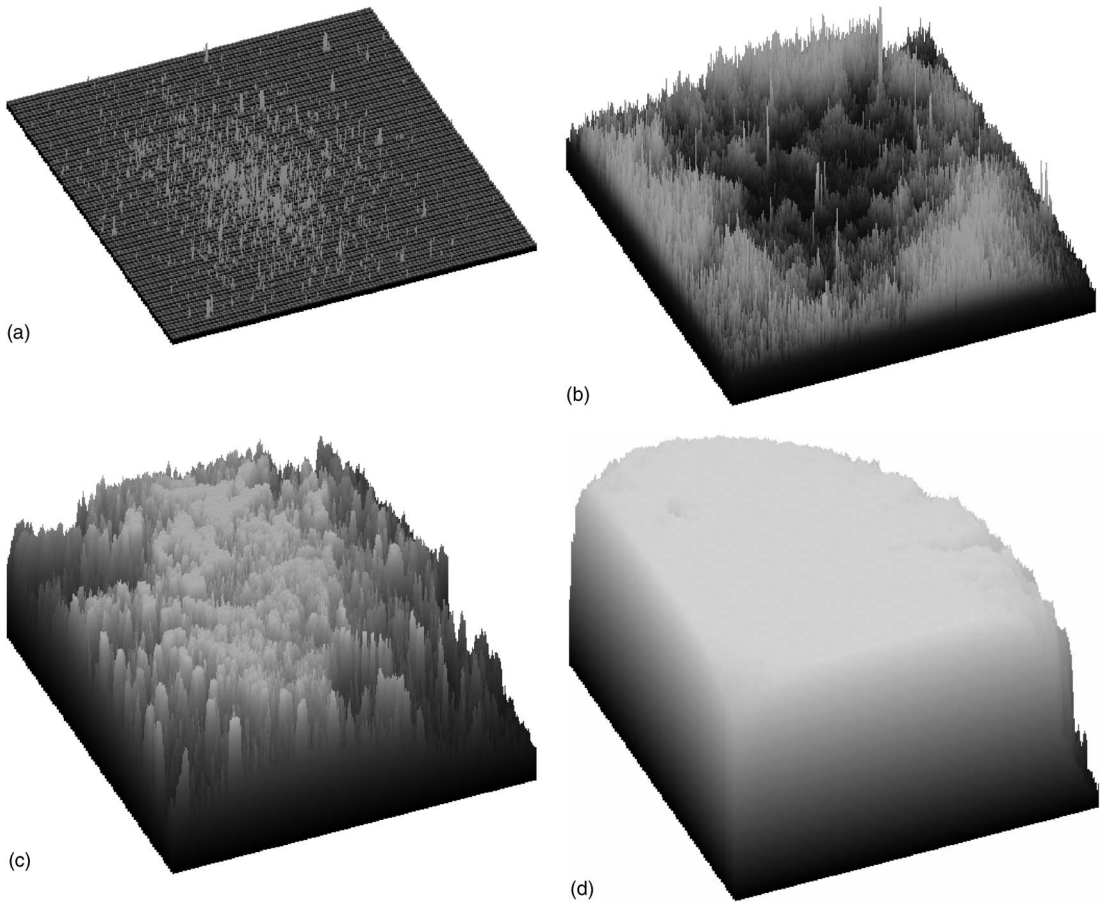


Fig. 3 Volume images of Bfm+ *E. faecalis* isolate on glass computer-rendered from 2-D confocal scanning laser microscope images obtained at times of (a) 2, (b) 8, (c) 12, and (d) 24 h.

stratum, reflectance measurements are made for four randomly chosen wells on a given substratum sample. The substratum is then rotated 90 deg and measurements are made again for four randomly chosen wells. The data is processed as explained in the next section, and the profiles are compared for anomalous behavior.

For the results presented in this work, reflectance measurements were stored for wavelengths between 200 and 800 nm, inclusive. Thus, each well scan resulted in 601 data points. Scan files for each substratum were saved as comma-delimited text files and processed as described next.

3 Biofilm Experiments

Environmental isolates of *Enterococcus faecalis* and *Enterococcus faecium* were obtained from the Field Disease Investigation Unit (Washington State University, Pullman, Washington). Two strains, identified as biofilm forming (Bfm+) and nonforming (Bfm−) using a crystal violet assay,[9] were grown in tryptic soy broth (TSB) supplemented with 0.125% glucose. Bacteria were grown overnight with agitation and diluted at approximately 1:40 in fresh media. Cell dilutions (35 μl) were spotted onto sterile 12-well, Teflon-masked glass slides (Erie Scientific, Portsmouth, New Hampshire) with four wells of Bfm+ and four of Bfm− strains. In addition, four control wells were used that contained media but no cells. These wells served as controls for contamination and were also used to calibrate the reflectance measurements. Cells were allowed to form biofilm statically at 37°C for 2, 4, 8, 12, and 24 h in a humidity chamber. At each time point, unattached cells were dislodged by successive 1-min washes with submersion of the slide into 50% and 95% ethanol; this also served to fix the biofilm on the slide surface. The slides were then air dried at room temperature. To determine any problems with the submersion washing technique, a more time-intensive approach was tried. A duplicate set of glass slides was prepared, and a single channel pipette with sterile barrier tips was used to draw off the liquid with unattached cells from each well and to add 35 μl of 50% ethanol, which was removed after 1 min. This was repeated with 95% ethanol. The differences in reflectance results were insignificant. Note that fixation with ethanol preserves the cells without deforming them, but it is not required for reflectance measurements. In this study it was used to dehydrate the cells for imaging and long-term storage. The procedure used to grow biofilms on a polystyrene substratum (Costar, Corning, New York) was identical to the one for glass, except that 96-well plates were used.

Reflectance measurements were obtained for all 12 wells on each substratum at all time points. The results for each set of four wells were averaged to obtain reflectance values for

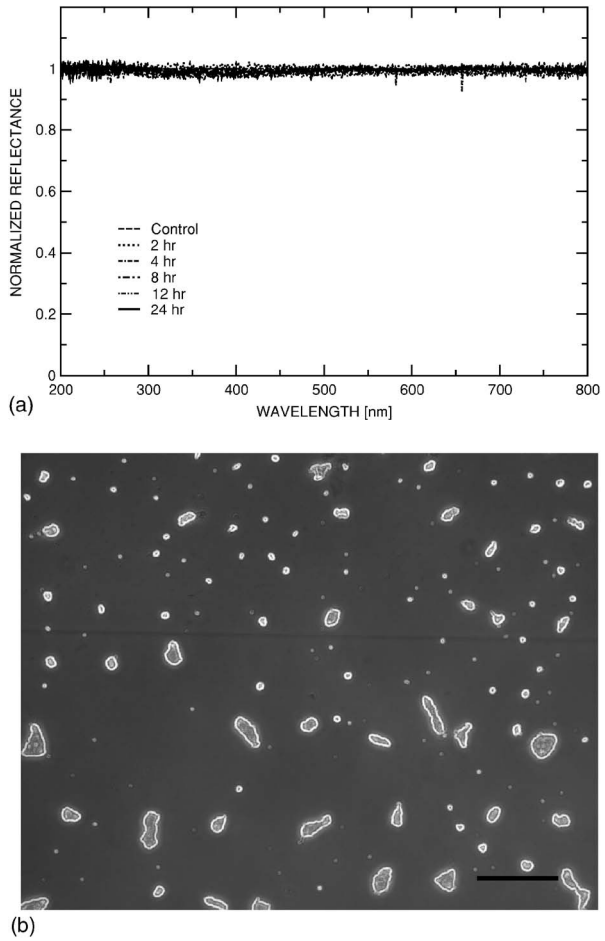


Fig. 4 (a) Normalized reflectance measurements as a function of wavelength for Bfm− *E. faecium* isolate on glass at times $t=0$ (control), 2, 4, 8, 12, and 24 h. (b) Phase contrast image of this isolate after 24 h of growth on glass. The scale bar is 50 μm.

each strain at all 601 wavelengths. The averaged control values for a given time point, substratum, and wavelength were used to normalize the data. This included the control values themselves, resulting in a constant normalized reflectance value of one for the control. For the clean substratum samples used to examine surface anomalies, the same approach was used except that the results were not normalized.

As mentioned earlier, control wells were used for reflectance calibration. It is necessary to measure the reflection from each substratum without any biofilm to obtain the control values used for normalization. This method of calibration insures that reflectance values for the different bacterial strains on a substratum are relative to that actual substratum, not just the type of substratum.

Development of the *E. faecalis* Bfm+ strain used in this study was assessed at varying incubation times on Teflon-masked glass slides at 37°C. After reflectance measurements were obtained, the biofilm was subsequently stained with 1% aqueous solution of acridine orange (Sigma, Saint Louis, Missouri) for 15 min and rinsed twice with sterile water. A Z series of images of biofilm was collected in 2-μm increments starting from the glass surface using an MRC 1024 confocal scanning laser microscope (Biorad, Hercules, California) equipped with a krypton/argon laser connected to a Nikon Eclipse TE 300 compound light microscope with a 40× objective. Semiquantitative estimates of an approximate area of 20×20 μm^2 were collected for surface coverage and biofilm volume measurements. Triplicate independent regions of each surface were collected, and a 3-D image was rendered from the Z-series images using Image J software (National Institutes of Health, http://rsb.info.nih.gov/ij/).

4 Results

Figure 2(a) shows reflectance results for the Bfm+ *E. faecalis* strain on glass at $t=0$ (control), 2, 8, 12, and 24 h (4 h is not shown to simplify the plot). Cell attachment is identified by saturation of the reflectance at the lower wavelengths (<300 nm) over time. At 2 h, attachment is incomplete. The values of the reflectance at the lower wavelengths at 8, 12, and 24 h are essentially the same, indicating that attachment was complete by 8 h. As the biofilm grows in volume over time, the reflectance first decreases over all wavelengths (8 h) and then increases at higher wavelengths (12 h, 24 h). The development of a 3-D biofilm for the same isolate and time points was verified using confocal scanning laser microscopy (Fig. 3). The phase contrast microscope (PCM) image of the Bfm+ *E. faecalis* isolate after 24 h of growth also shows a fully formed biofilm [Fig. 2(b)]. Note that spikes in the reflectance data at 435 and 655 nm in Fig. 2(a) are due to the instrument light sources. The remaining spikes are due to inadequate warming of the power supply.

Reflectance measurements for the *E. faecium* Bfm− strain are shown in Fig. 4(a). The profiles at all time points differ substantially from those of the Bfm+ strain. There is no significant difference between the control data and the data for the bacteria at all time points. The lack of a decrease in the reflectance indicates that no biofilm growth occurred as confirmed by PCM imaging [Fig. 4(b)].

Reflectance profiles for biofilm formation are substratum dependent, but consistent results have been obtained for repeated experiments with the same substratum. Figure 5(a) shows reflectance results for the Bfm+ *E. faecalis* isolate on both glass and polystyrene at $t=24$ h. Both profiles show the steep decline in reflectance at lower wavelengths indicative of attachment and the continuing low level of reflectance that signals a fully formed biofilm. Overall the difference between the reflectance profiles is minimal. The fully formed biofilm on polystyrene is confirmed by PCM imaging [Fig. 5(b)]. As with the glass substratum, the Bfm− *E. faecium* isolate did not form a biofilm on polystyrene [Figs. 5(c) and 5(d)].

Development of the reflectance assay was motivated by the desire for a rapid means of quantifying biofilm growth on multiple substrata. For the two substrata presented in this work, a simple averaging technique discussed in Ref. 16. leads to a single number that indicates whether or not a biofilm has formed. The normalized reflectance values at 24 h for wavelengths between 250 and 800 nm are summed, and the total is divided by the number of values summed. This average is subtracted from 1 giving a value between 0 and 1, with 0 equivalent to total reflection and 1 equivalent to no reflection at all. From empirical studies of 14 different *Enterococcus* isolates, values less than 0.15 indicate Bfm− *Enterococcus*, and values above 0.5 indicate Bfm+ *Enterococcus*.[16]

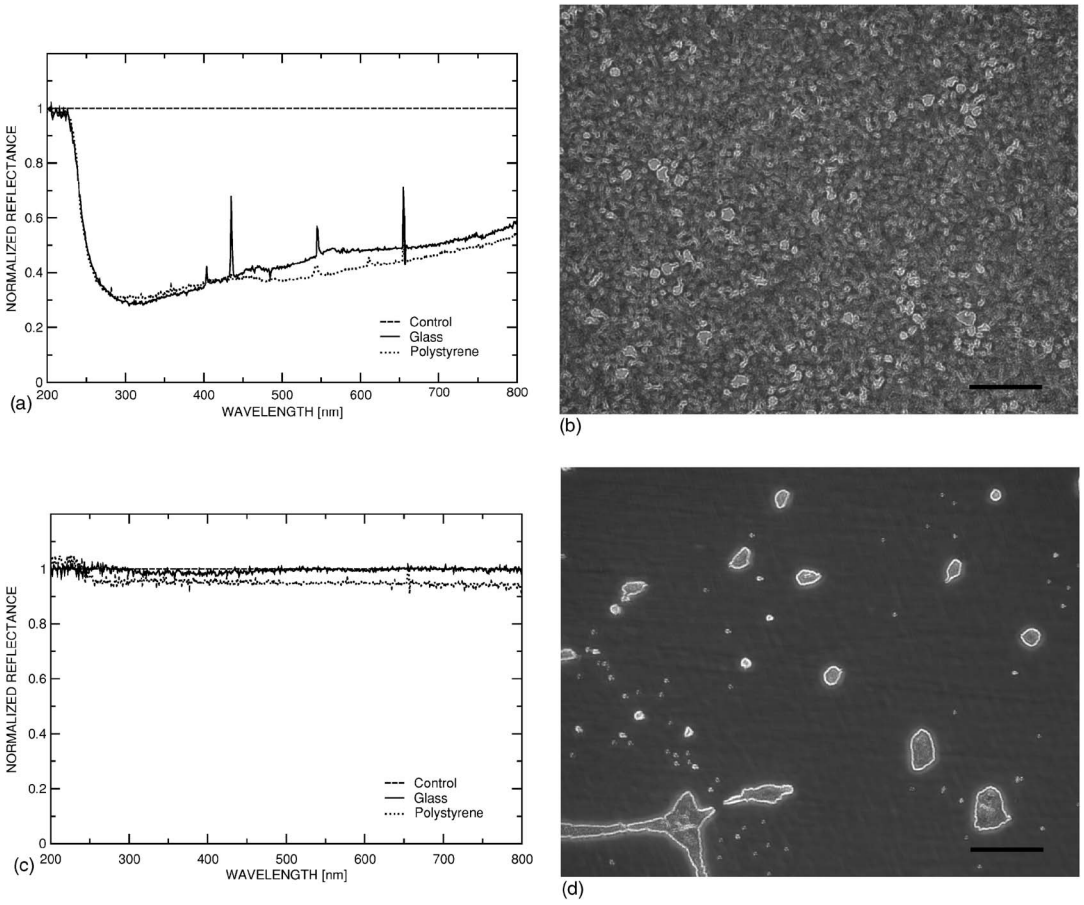


Fig. 5 (a) Normalized reflectance measurements as a function of wavelength for Bfm+ *E. faecalis* isolate on glass and on polystyrene for biofilm grown for 24 h. (b) Phase contrast image of this Bfm+ isolate after 24 h of growth on polystyrene. (c) Normalized reflectance measurements as a function of wavelength for Bfm– *E. faecium* isolate on glass and on polystyrene grown for 24 h. (d) Phase contrast image of the Bfm– isolate after 24 h of growth on polystyrene. The scale bars are 50 μm.

Whether this algorithm holds true for other substrata and other bacteria is the subject of further research. If it does not, however, it is probable that a similar algorithm can be determined empirically.

5 Discussion

Methods for quantifying biofilm formation typically rely on enumerating bacteria attached to a surface. Early techniques of labeling cells with radioisotopes, colony counts of cell suspensions recovered from surfaces, and staining attached cells using dyes such as crystal violet and acridine orange[17,18] have evolved into a streamlined, high-throughput approach using 96-well polystyrene microtiter plates for both the development and detection of biofilm formation using crystal violet as the indicator dye.[19,4] We have described a new approach that is based on the optical properties of biofilm formation. Rather than determining the relative differences in a cell population on a surface, the reflectance assay monitors the changes in optical reflectance to measure biofilm formation.

For a clean, media-free substratum, some light will be reflected by the surface and some will be transmitted into the substratum. By the law of reflection, if light is normally incident onto a flat substratum, it will be reflected in the same direction [Fig. 6(a)].[20] In theory, for a clean substratum ($t=0$ h) this reflection is independent of wavelength, but in reality the measured values vary by wavelength as a result of the characteristics of the spectrometry equipment. Because the light intensity transmitted and received is not uniform at all wavelengths (the sharp discontinuities in the reflectance plots are artifacts of the equipment), it is necessary to normalize the measured values to those of the values for the control.

After introduction of the bacterial cells and media to the wells, the cells begin to adhere to the substratum. Reflectance measurements decrease [e.g., see Fig. 2(a)] because the cells "roughen" the surface so that light is no longer reflected in one direction [Fig. 6(b)]. In addition, the cells themselves absorb some of the light energy. Both scattering and absorption are wavelength dependent as evident in Fig. 2(a). As the surface coverage increases, the reflectance further decreases because of the increased surface roughness, absorption by the cells, and absorption by the biomass accumulating on the surface. For this work, biomass is defined as all other components of a biofilm excluding cells, and thus refers to extracellular DNA, proteins, polypeptides, polysaccharide, cations, and other inorganic material. When a crude, cell-free extract of an *E. faecalis* biofilm is isolated[21] and applied to a clean glass surface, reflectance measurements decrease, corroborating that biofilm components other than cells affect reflectance

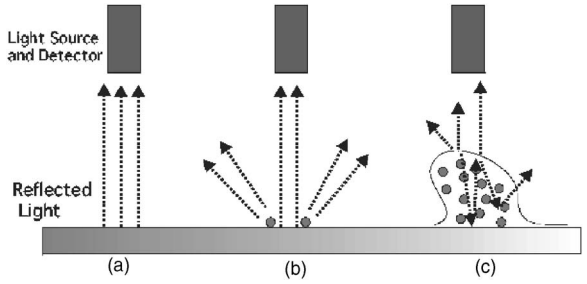


Fig. 6 Reflectance-assay model. (a) On a clean, flat substratum, incident light is reflected directly back to the detector; (b) as cells multiply and attach to the substratum, reflectance levels decrease because of absorption and scattering by the cells; (c) as a biofilm develops, reflectance further decreases due to multiple scattering within the biofilm layer, both at the interfaces of the biofilm and between the cells themselves, as well as to increased absorption by the cells. For a fully formed biofilm, the reflectance actually increases at higher wavelengths as more light is scattered back to the detector.

measurements (data not shown). While it is not yet possible to use reflectance to discriminate between the specific components of biofilm, the results from this study show that measuring surface reflectance is an effective means of monitoring biofilm formation.

The increasing thickness of the developing biofilm leads to another source of reflectance loss in the form of scattering within the biomass. A biofilm is a heterogeneous mixture of cells and biomass that is approximately 95% water.[22] As a biofilm increases in depth, the turbidity of the biofilm mixture increases. As turbidity increases, reflectance decreases. Light enters the biofilm layer and is scattered between cells and biomass contained within the layers of biofilm. These interactions lead to greater absorption and multiple scattering of light, resulting in less light returned to the detector [Fig. 6(c)]. As the biofilm continues to thicken and increase in volume, the scattering and absorption mechanisms further change. At 24 h when the biofilm is fully developed, reflectance actually increases somewhat over the visible and infrared regions (400 to 800 nm).

6 Summary

We develop an inexpensive, nondestructive optical reflectance assay for measuring biofilm formation on both opaque and nonopaque surfaces. Time series reflectance measurements are presented for two different bacteria on two different substrata, glass and polystyrene. These measurements distinguish between attachment and volume growth and, when averaged over wavelength, provide a quantitative value that indicates whether a bacterium is a biofilm "former" or "nonformer." This permits use of the reflectance assay for rapid quantitative screening of mutant libraries, as well as rapid assessment of phenotypic variation among isolates. Finally, while biofilm specimens need not be fixed, this procedure can be used to preserve samples for future examination. Images have been obtained of specimens stored for six months with no apparent degradation.

Acknowledgments

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