APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1997, p. 4600–4603 Vol. 63, No. 11
0099-2240/97/$04.00+0


# Combined Light Microscopy and Attenuated Total Reflection Fourier Transform Infrared Spectroscopy for Integration of Biofilm Structure, Distribution, and Chemistry at Solid-Liquid Interfaces

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Received 18 March 1997/Accepted 8 July 1997

**Reflected differential interference contrast microscopy and attenuated total reflection Fourier transform infrared spectroscopy were used to obtain complementary data on the structural and chemical properties of a biofilm. This information was obtained nondestructively, quasisimultaneously, and in real time, thereby permitting the verification of time-dependent relationships between the biofilm's population structure, distribution, and interfacial chemistry. The approach offers opportunities to examine these relationships on a variety of substrata in the presence of a bulk aqueous phase under controlled hydrodynamic conditions.**

The evaluation of microbial processes at surfaces has, to a large extent, been dependent upon microscopic examination, indirect analysis of bulk aqueous-phase properties, or analysis of the biofilm following its removal from the substratum. While these techniques provide useful information on colonization dynamics, biofilm structure, and the impact of biofilm processes on the adjacent bulk aqueous phase, they offer little insight into the chemistry of the biofilm matrix or the underlying substratum surface. The chemistry of intact biofilms has been characterized with microelectrode probes (3) and by microscopic observation of a variety of fluorescent markers (6, 13). Spectroscopic techniques provide a means of evaluating the chemistry of biological processes nondestructively, in real time, and with negligible perturbation of the sample. Techniques which utilize the evanescent wave to enable surface sensitivity are perfectly suited to the investigation of attached cells (14). Infrared (IR) spectroscopy has been used to characterize the biochemistry of bacteria (8, 17). Attenuated total reflection Fourier transform IR (ATR/FT-IR) spectroscopy has been used to evaluate the chemical changes that occur at the bulk aqueous-phase–substratum interface during biofilm development (9, 10, 16), but it offers little opportunity to relate these chemical changes to biofilm architecture. We describe here an integrated microscopic–ATR/FT-IR experimental system whereby changes in biofilm parameters that are resolved and quantified by light microscopy and computer-controlled image analysis can be related to surface area-averaged chemical changes at the biofilm-substratum interface.

A flat-plate flow chamber, based on a previously described design (2), was modified to fit into a Horizon Cell mirror assembly (Harrick Scientific, Ossing, N.Y.) mounted on the optical bench of a Nicholet 740 FT-IR spectrometer (Fig. 1). A glass window installed in the top of the flow chamber permitted microscopic examination with reflected differential interference contrast (DIC) optics of the surface of a 50- by 10- by 2-mm germanium (Ge) trapezoidal prism (Harrick Scientific) which formed the base of the flow channel. The flow channel was approximately rectangular (45 by 10 by 0.75 mm), with liquid entrance and exit ports at each end of the longest side. The flow chamber was designed so that it could be shuttled between the Horizon Cell in the FT-IR spectrometer and the stage of an Olympus BX60 microscope equipped with reflected DIC optics and a long-working-distance 40× water immersion lens (Nikon), allowing bacteria attached to the Ge prism to be resolved through the flow chamber viewing window and the aqueous medium without being stained and without removal of the coverslip. A specially designed cavity in the top of the Horizon Cell mirror assembly maintained the alignment of the prism in the optical path of the IR beam each time it was shuttled to the spectrometer from the microscope stage.

The Ge prism and flow chamber were cleaned as previously described (5). The flow chamber was assembled and sterilized by soaking in 70% ethanol for 3 h. It was subsequently filled with sterile culture medium (see below) from a medium reservoir through the use of sterile silicone tubing and a peristaltic pump and mounted in the Horizon Cell in the FT-IR spectrometer, and a background IR spectrum of the aqueous medium was collected. All IR spectra were converted to absorbance spectra with instrument software.

An undefined mixed culture of bacteria isolated from human saliva was grown in batch at 37°C with aeration in a 1% glucose–tap water solution. Upon entry into stationary phase, the culture was pumped through the flow chamber and over the surface of the Ge prism, replacing the sterile culture medium at a flow rate of 1 ml/min for 1 h, which permitted bacterial colonization of the prism under the flow. The bacterial culture was then replaced by a continuous flow of sterile culture medium through the chamber to promote growth and biofilm development by the bacteria that had attached to the prism.

Reflected DIC light microscopic images of the bacteria that had attached to and colonized the prism surface were collected at various times after chamber inoculation. Corresponding microscopic images and IR spectra of the wetted prism surface were collected within 2 min of each other during bacterial colonization. Images were captured and digitized with a black-and-white, computer-controlled Photometrics charge-coupled

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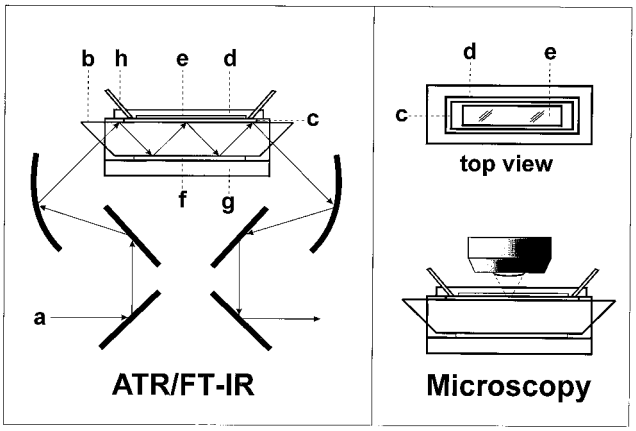


FIG. 1. Schematic of the flow chamber. The beam (a) is guided by a series of mirrors (Harrick mirror assembly) into the Ge prism (b) where it reflects internally. The viton spacer (McMaster-Carr, Los Angeles, Calif.) (c) is held against the Ge prism by a stainless steel cover (d). A coverslip (e) glued to the cover allows microscopic observation. A Silastic spacer (f) covered with aluminum foil cushions the prism against the holder (g). The entire prism-holder-cover assembly can be transferred to the microscope and back to the Harrick mirror assembly. The flow cavity was formed by the inner surfaces of the viton spacer, the Ge prism, and the coverslip. Medium is introduced to the flow cavity via entrance (h) and exit tubing gold soldered to the stainless steel cover.

device camera with Image-Pro Plus software (Media Cybernetics, Silver Spring, Md.). Bacteria were enumerated by counting 100 to 500 bacteria within a known area demarcated by the image analysis software. During the 10-h period after inoculation, the prism surface became increasingly colonized by bacteria which displayed different cell morphologies (Fig. 2a to c). Another type of biofilm heterogeneity, in the form of aggregates of cells piled on top of each other, was also resolved at the end this time period (Fig. 2c). Passage of an air bubble through the flow chamber resulted in a decrease in cell density (Fig. 2d).

Difference spectra, obtained by subtracting the background spectrum from the spectrum collected at each microscopic observation period, yielded the IR spectrum contributed by the microorganisms colonizing the substratum at that point of biofilm development (Fig. 3). The difference spectra displayed features which originated from various biopolymers (16). The bands at approximately 1,650 and 1,550 $cm^{-1}$ originated from proteins (amide I and II bands). Features with peaks at approximately 1,240 and 1,080 $cm^{-1}$ originated primarily from phosphodiester linkages of RNA and DNA. Polysaccharides exhibited bands in the region from 1,130 to 900 $cm^{-1}$, with a prominent C–O bond stretch at approximately 1,030 $cm^{-1}$. The amide II band area was approximately proportional to the

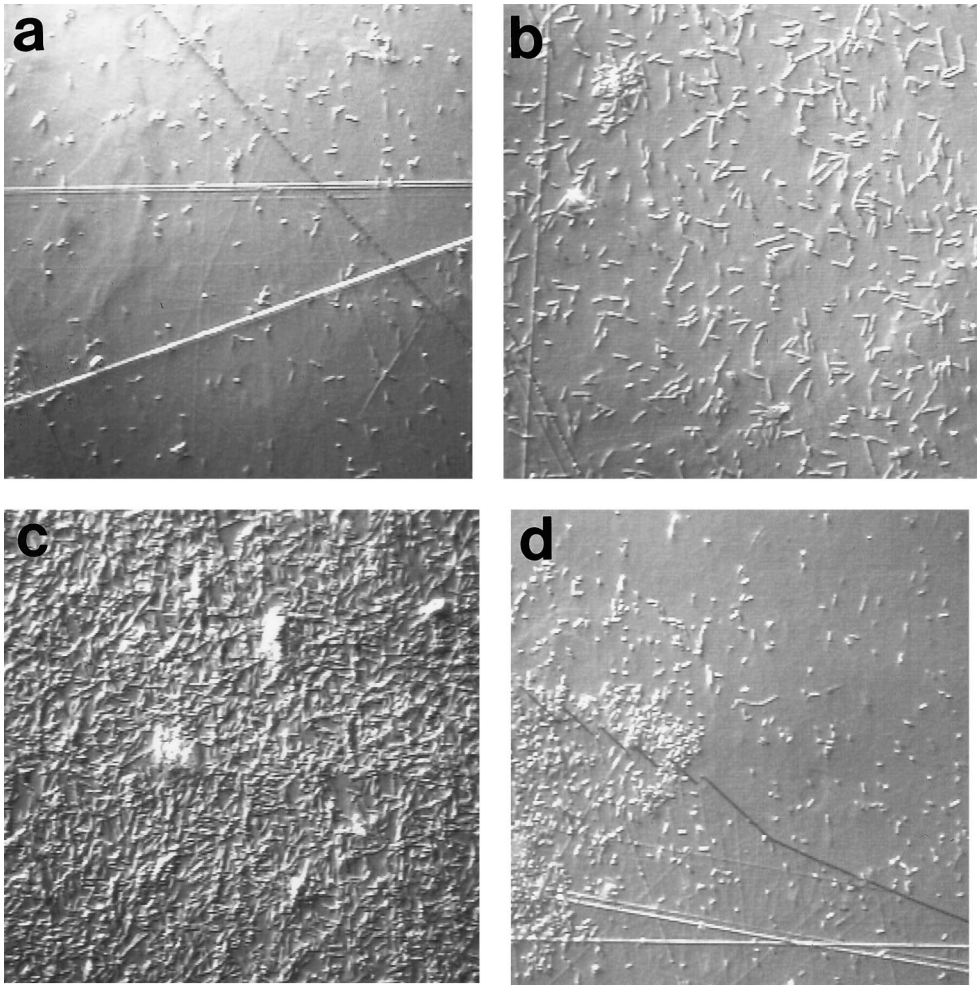


FIG. 2. Micrographs of bacteria colonizing the surface of the Ge prism after 1 h (a), 5 h (b), and 10 h (c), and 1 h after passage of an air bubble through the chamber (d).

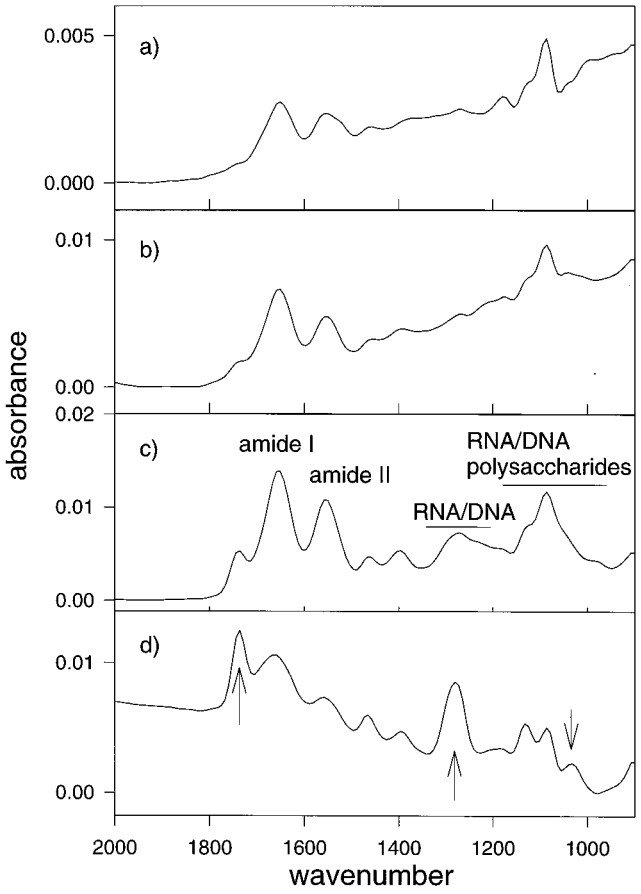


FIG. 3. FT-IR spectra corresponding to the micrographs in Fig. 2. Regions of prominent bands originating from biopolymers are indicated in panel c; bands originating from putative acidic polysaccharide are indicated in panel d.

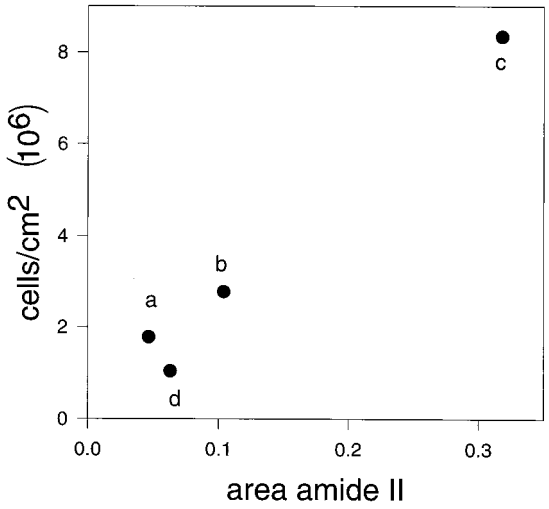


FIG. 4. Cell density versus area of the amide II band. Letters refer to the micrographs in Fig. 2.

density of bacteria colonizing the surface (Fig. 4). Although this relationship is expected theoretically, this is first time it has been verified by direct observation.

The removal of attached bacteria from the prism surface that occurred as a result of passage of the air bubble through the chamber led to a change in the shape of the FT-IR (compare Fig. 3c and d). Bands at approximately 1,730, 1,250, and 1,030 $cm^{-1}$ became more prominent after bacteria were removed from the surface. A relationship between this IR spectrum change and cell detachment would have been difficult to establish by other analytical approaches. Bands in approximately the same positions have been shown to originate from an acetylated bacterial alginate (4). The adhesive portion of a biopolymer incorporating similar chemical groups may have remained on the substratum after the cells were removed. Analysis of such biomolecular remnants has been proposed as a means of characterizing nonspecific adhesins (11). The combination of ATR/FT-IR spectroscopy and microscopy lends itself to investigation of these types of phenomena.

In summary, it is feasible to combine microscopy and ATR/FT-IR spectroscopy to characterize the chemistry and architecture of biofilms colonizing surfaces in vitro. The depth of penetration of the evanescent wave of IR radiation restricts the region over which chemical information is obtained to within approximately 1 μm of the surface of the prism. This feature has been exploited recently to evaluate the transport kinetics of antimicrobial agents within undisturbed biofilms (16). With the approach described here, it should now be possible to relate specific structural changes reported for antibiotic-challenged biofilms (7) to the concentrations of antibiotics within the biofilms. Through the application of thin-film technologies, ATR/FT-IR spectroscopy can be extended to the study of biofilms colonizing a variety of substrata. Ultrathin films of hydroxyapatite (15), synthetic and naturally occurring polymers (1, 5), and alloys of metals (12) have been deposited on Ge prism surfaces to study interactions between these materials and microbial biofilms. Incorporating ultrathin-film technology into the experimental system described in this paper would offer opportunities to relate biofilm matrix chemistry to biofilm population distribution and architecture for the investigation of fouling of surfaces in the oral cavity, prosthetic devices, synthetic membrane materials, and metal conduits.

This work was supported by a grant from The Whitaker Foundation (RG 94-0256) (P.A.S.) and by a grant from the National Institute of Dental Research (N01-DE-62611) (R.J.P.). We also acknowledge support from National Science Foundation grant EEC 8907039.

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